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Postexcitatory Depression of Gustatory Receptors

By

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Received 8 January 1968

Abstract

Hellekant, G. *Postexcitatory depression of gustatory receptors.* Acta physiol. scand. 1968. 74. 1—9.

It was observed that when the same sapid solution was applied two or more times to the tongue of the cat, the first exposure depressed following responses although the tongue was continuously rinsed with water between the periods of stimulation. This depression vanished gradually but was noticeable after some substances even after one min of water rinse. The time course of the depression depended on the chemical and its concentration, but seemed to be unaffected a) by changes in temperature (27—40° C), b) by the duration of stimulation (3—10 sec) and c) the flow rates of the water rinse (1.7—5 ml/sec).

In an earlier series of experiments which involved repeated exposures of the tongue to a given chemical, it was observed that the second application frequently elicited a reduced response, despite the fact that the tongue was rinsed with water for several sec between the applications. Thus it seemed as if the first exposure depressed the response to the following without observable changes in the base line activity.

An investigation of the recovery of taste receptors from the effects of prior stimulation may therefore be of interest both from the standpoint of the biophysical mechanisms of taste stimulation and for developing proper methods of taste stimulation.

Methods

Thirteen cats were used. The animals were anaesthetized with sodium pentobarbitol (Diabutal) 50 mg/ml. The initial dose was administered i.p. and sustained with i.v. doses. The lateral approach described earlier (e.g. Cohen *et al.* 1955) was used when dissecting the chorda tympani nerve. Recordings from the whole nerve were obtained with a silver electrode fixed in a micromanipulator. The nerve potentials were amplified by a Grass AC preamplifier, summated by a summator circuit described earlier by Diamant *et al.* (1965) and recorded by a Sanborn DC writer model 67 1200.

The method of temperature control and application of solutions was described by Hellekant (1965). The temperature of water and solutions was kept at 27° C except when the effect of higher temperature was tested. The stimuli were applied for about 3 sec except when the effect of prolonged stimulation was studied. The solutions were made up in distilled water which also was used as stabilizing and removing rinse that flowed constantly (5 ml/sec) between stimulations. Thirteen different chemicals were used: $CaCl_2$, CH_3COOH (HAc), choline chloride, HCl, KCl, K_2SO_4, LiCl, Li_2SO_4, $MgCl_2$, NaCl, $NaNO_3$, NH_4Cl and quinine hydrochloride. Their concentrations were chosen in order to elicit approximately the same response in the chorda tympani nerve. Table I gives the concentrations used.

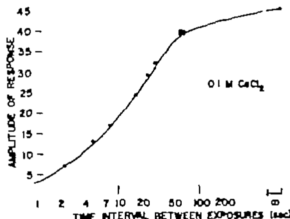


Fig. 2. The amplitude of each response in the previous figure is plotted versus the logarithm of time interval since the preceding stimulus, giving the RRF of the solution.

if the function was growing with increasing, decreasing or linear slope as these characteristics depended on the time scale used. They were all growing with decreasing slope when plotted against a linear time scale.

The RRF d pendenc of hemical

The duration of depression differed with the chemical used. The recovery after NH_4Cl, acids and quinine hydrochloride was rapid. Th recovery after potassium salts was somewhat slower The recovery to choline chloride sodium chloride, lithium salts, $CaCl_2$, $MgCl_2$ and $NaNO_3$ was slower in the order mentioned. This can be seen in Table I where a time constant τ was introduced and listed. This concept is used despite the fact that the RRF plotted versus the logarithm of time did not always display a straight line was therefore simply defined as the time in sec when 2/3 of maximum response was reached. The first row in Tabl I shows the average time constant $(\bar{\tau})$ f r each solution, the second the number of animals in the value (n) the third th shortest time constant observed for th solution (τ_{min}) and the fourth the largest one (τ_{max})

There was a great deal of variation among the animals. The τ value varied less when a stimulus was applied several times to the same animal The average variation was then 17 per cent, S.E. 3 per cent. It was also observed that the sequence of solutions when arranged according to their τ values was the same n almost all nimals, though their values differed.

Th RRF d penden e of the i tervening water rinse

It might be surmised that the observations described so far could be attributed to an incomplet removal f stimulus from the t ngue Thus the diminished response might result from a concentration difference between remaining and new solution. An experimental attempt was made to test this possibility by diminishing the flow rate of the intervening water rinse. This would then cause a slower recovery since the sapid solution would be more slowly removed. Seventeen test eries were run

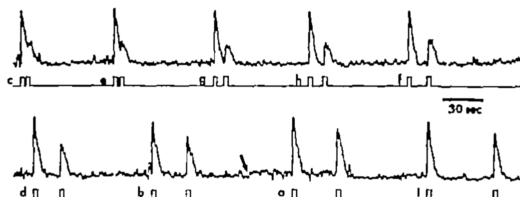


Fig 1 show series of summated responses to 0.1 M $CaCl_2$ solution in one cat. The actual order of presentation is indicated by the letters. Upward deflection of signal shows the onset of stimulus, downward the onset of water Flow rate 5 ml/sec

Results

Fig 1 shows a series of responses to 0.1 M $CaCl_2$ solution. The recording was obtained from one animal during a single experiment. The series of 9 pairs of different intervals was presented in a random sequence For clarity the record was rearranged to show the effect of increasing intervals between the first and second stimulation. The actual order of presentation is indicated by the letter a—i under the first of each stimulus pair The response to the right of the letter a in Fig 1 was recorded during the first exposure to $CaCl_2$ It can therefore be regarded as the response after an infinit interval, thus showing the maximum response under this recording condition It is evident from Fig. 1 that the height of the second response grew with the interval from the preceding exposure

The arrow in Fig 1 was placed at the point where the baseline before CaCl exposure is adjacent to that after four exposures. The baseline after four exposures seems to be smoother Such a difference was observed in several cases and can not be attributed to a difference in amplification, but must be regarded as another lasting effect caused by the CaCl solution.

The amplitude of each response in Fig 1 was measured and plotted versus the logarithm of time interval ince the preceding stimulus. This f nction can be seen in Fig 2. It was observed throughout this series that similar relationships could be obtained in all the animals to all chemicals though, as will be shown below the time course of the functions aried and for MgCl and CaCl the initial phase of the curve was different when plotted in this manner from that obtained to the other chemicals. These curves describe what will be called the receptor recovery functions (RRF) in the remaining part of this study

The initial phase of the curve in Fig 2 with its continuously increasing slope differed from those obtained to other chemicals, except for $MgCl_2$ in some recordings. The initial phase of the RRF to other chemicals was always growing linearily or with a continuously decreasing slope. However it may be of rather little importance

stimulus. Competition between stimuli occurs if they are presented together and resemble each other from the receptor's point of view. Experimental observations have shown a relatively fast reaction between most stimuli and their receptors. Less than one sec if the duration of the transient response is used as a measure (Zotterman 1959). It is conceivable that the rate of disengagement between receptor sites and stimulus is slower than the rate of engagement i.e. the affinity between sites and stimulus is larger than between water and stimulus. Further the affinities between stimuli and their sites differ (Beidler 1962). It is therefore rather obvious that the rates of disengagement between stimuli and their sites may differ. This would be observed as a variation of the RRF between the chemicals.

It has also been observed that the duration of the postexcitatory depression is lengthened when the duration of stimulation is extended (*cf.* Granit 1955 p. 28 ff.). This is in contrast to the observations in this study which indicated that an extension of stimulation time from 3 to 10 sec had no effect on the postexcitatory depression (RRF). Beidler' general taste equation (1954) was derived from the concept of an obtained equilibrium between the number of unoccupied and occupied receptor sites. This concept can be used here if it is assumed that the reaction between stimulus and its sites reaches equilibrium within 3 sec. Consequently extended stimulation should not increase the number of occupied sites and would have no effect on the recovery after stimulation. The time course of the electrical recorded response during stimulation with the substances used in this study indicates also that the adsorption between stimulus and its receptor sites had reached an equilibrium within 3 sec, as the electrical response then had reached a steady or slowly declining state. Three sec have also been shown to be long enough for the brain to experience and determine a gustatory sensation (Marcström 1967).

This study indicates an effect of different stimulus concentrations on the RRF as it was observed that higher concentration usually gave a smaller value. This may seem paradoxical as it is implicit in the adsorption theory that during stimulation a stronger concentration of stimulus will occupy a larger number of the available receptors sites than a weaker. Fewer receptor sites will also be available after stimulation with a stronger stimulus than after a weaker. This should be shown as a larger depression of the response after the stronger concentration provided that the same concentration of solution is used as second stimulus in both cases. But in the present series the strength of the second stimulus was not kept constant but increased to the same level as the first i.e. the same stimulus was repeated. This complicates the situation as the stronger depression, caused by the increase of strength of the first stimulus, will be counteracted by the increase of strength of the second stimulus. In this study the r values in general became smaller when both concentrations were raised which may indicate that the increase of stimulus strength during the first 2/3 of the recovery overruled the increased inexcitability. A study in which the concentrations of the first and second stimulus were changed independently and not as in this study simultaneously may clarify the situation.

The influence of temperature on the adsorption between a salt stimulus and its

receptor sites is small when judged by the neural response (Beidler 1953) especially in the temperature range 30 to 40° C (Yamashita *et al* 1964) The effect of temperature changes in the rate of disengagement between stimulus and its receptor sites is probably also small and therefore not noticeable within this temperature range. It may therefore explain the absence of an effect on the RRF when the temperature was raised from 27 to 40° C.

The possibility that unbound residues of stimulus were responsible for the observations described in this study must also be considered. It has been shown that stroking (Appelberg 1958, Kitchell 1963) and stretching (Ishiko and Amatsu 1964) the tongue enhance the gustatory response recorded from the glossopharyngeal nerve. Probably because they open the moats around the vallate papillae and give stimulus access to the taste buds in this part of the tongue. It is therefore possible to suspect residues in the grooves around the papillae containing the taste buds from which recordings were made in this study However the anatomical features are not the same in the anterior part of the tongue as in the posterior Its taste buds are localised in the walls of the fungiform papillae (Hayes and Elliott 1943) These papillae are situated more superficially and the clefts between them are more shallow Gustatory stimuli as well as water have therefore easier access to the pores of the anterior taste buds which are supplied by the chorda tympani nerve. Ishiko and Amatsu found no effect of stretching the tongue on the response of the chorda tympani nerve. Further the experiments on which the water flow was decreased m 5 to 2 ml/sec did not affect the recovery after stimulation as it certainly would done if residues on the tongue were responsible for the depression.

Each taste bud is composed of several gustatory cells which by means of microvilli project into the taste pore. The taste pore is open to the solution around the papilla. Ultramicroscopical observations by Trujillo-Cenóz (1957) De Lorenzo (1963) and Nemetscheck-Gansler and Ferner (1964) show that microvilli do not project outside the taste pore The last mentioned authors also state that there is a space between the mouth of the taste pore and the microvilli. Beidler suggested (196.) that the taste stimulus within the taste pore is transported to and from the membrane of the microvilli by diffusion. He estimated the importance of diffusion as small when stimulating But because of the time course of diffusion a low level or threshold concentration will take longer to achieve when stimulus is removed than when it is applied. A comparison between the order of diffusion velocities of the substances used and their RRF is therefore of value when evaluating this factor The order of diff sion velocities of the inorganic salts (Renqvist 1919 Handbook of Chemistry and Physics 1965 p F-43) agreed with the order of recovery after stimulation in the present experiments, except for $NaNO_3$ which showed the slowest recovery curve although its calculated diffusion velocity is relatively fast. However choline chloride and quinine chloride have substantially lower diffusion velocities than the other substances but the recovery after these two was among the fastest. It is therefore not very likely that the time for diffusion is the main factor in the recoveries observed.

Finally it may be suggested that the decrease of excitability studied here, can be better described as a stage of extended adaptation. However the term postexcitatory depression of excitability may be more adequate as sensory adaptation has been described as the decline in the frequency of discharge of an afferent fibre coming from an endorgan which is being subjected to continuous stimulation (Granit 1955 p. 24). Adaptation occurs during stimulation and postexcitatory inexcitability is observed after stimulation.

The experiments to this study were carried out at the Research Laboratory of Electronics, Massachusetts Institut of Technology Boston, Mass., U.S.A. It was supported by grant from Svenska Maltdrycksforskningsinstitutet.

References

AKAIKE, N and K. YAMADA, The depressant effect of calcium ions on the taste response of the frog. *K maned m d. J* 1965. *18* 169—170

APPELBERG, B., Species differences in the taste qualities mediated through the glossopharyngeal nerve. *Act physiol. scand* 1958. *44* 129—137

BEIDLER, L. M. Properties of chemoreceptors of tongue of rat. *J Neu physiol.* 1953 *16* 595—607

BEIDLER, L. M. A., A theory of taste stimulation. *J gen Physiol.* 1954 *38* 133—139

BEIDLER, L. M., Taste receptor stimulation. *Prog Biophys. biophys. Chem* 1962. *12* 107—151

COHEN M. J., S. HAGSTROM and Y ZOTTERMAN, The response spectrum of taste fibres in the cat: A single fibre analysis. *Act physiol. scand* 1955. *33* 316—332.

DE LORENZO, A. J D Studies on the ultrastructure and histophysiology of cell membranes, nerve fibers and synaptic junctions in chemoreceptors. *Olfaction and Taste* I. 1963 5—18.

DIAMANT H., B. OAKLEY L. STROM C. WELLS and Y ZOTTERMAN A comparison of neural and psychophysical responses to taste stimuli in man. *Act physiol. sc d* 1965. *64* 67—74

FUNAKOSHI, M. and Y ZOTTERMAN Effect of salt on sugar response. *Acta physiol scand* 1962. *57* 193—200.

GRANIT R., Receptors and sensory perception. Yale University press. 1962.

HANDBOOK of CHEMISTRY and PHYSICS, Cleveland, Ohio, 1964—1965.

HAYES, E. R. and R. ELLIOTT Distribution of the taste buds on the tongue of the kitten, with particular reference to those innervated by the chorda tympani branch of the facial nerve *J comp Neurol.* 1943 *76.* 227—238.

HELLEKANT G., Electrophysiological investigation of the gustatory effect of ethyl alcohol. I The summated response of the chorda tympani in the cat, dog and rat. *Act physiol sc d* 1965. *64* 392—397

HELLEKANT G Action and interaction of ethyl alcohol and some other substances on the receptors of the tongue. *Olfaction and taste II* 1967 465—479

INOUE, N and M ASHIDA Effects of stretch of the tongue on taste responses in glossopharyngeal and chorda tympani nerves of cat. *K maned med J* 1964 *17* 5—17

KITCHELL, R L Comparative anatomical and physiological studies of gustatory mechanisms. *Olfaction and taste I* 1963 235—255

MARCSTRÖM, A. Studies on the connection between physchiochemical properties and stimulation abilities of some sweet and bitter compounds. *A k. Zool* 1967 serie 2 *19* N 22. 421—535

NEMETSCHEK-GANSLER, H. and H. FERNER, Über die Ultrastruktur der Geschmacksknospen. *Z. Zellforsch.* 1964 *63* 155—178.

RENQVIST Y Ueber den Geschmack. *Sk d Arch Physiol.* 1919 *38* 97—201

TRUJILLO-CENÓZ, O Electron microscope study of the rabbit gustatory bud *Z Zellforsch.* 1957 *46* 272—280

YAMASHITA, S., K YAMADA and M S o, The effect of temperature on neural taste response of cats *J p J Physiol.* 1964 *14* 505—514

ZOTTERMAN Y The response of the frog's taste fibres to the application of pure water *Act physiol. scand* 1949 *18* 181—189

ZOTTERMAN Y The nervous mechanism of taste *A n. N Y Ac d Sci.* 1959 *81* 358

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The Reflection of the Age, Tissue and Solubility on the Amberlite CG-50 Fractions of Denatured Collagens

By

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Abstract

Kulonen, E. and J. Pikkarainen. *The reflection of the age tissue and solubility on the Amberlite CG-50 fract ons of denatu ed collagens* Acta physiol. scand. 1968. 74 10—15

Collagen was extracted and purified from the skins of young nd f ll-grown guinea pigs and from the skins and tendons of calf and cow first with neutral salt solutions and acidic buffers in the cold and then the residue was gelatinized by stepwise heating. I the young animals the collagenous material which could be solubilized at +65 C already was increased markedly The arious denatured collagens were fractionated with Amberlite CG-50 columns, eluted with buffers of increasing ionic strength and pH and finally with sodium hydroxide. The first fraction, emerging at pH 5.50 was large in the samples from soluble collagen (in comparison to insoluble) in the extracts from insoluble collagens of young animals (in comparison to adults) and in the collagenous fractions of tendon (in comparison to the skin) The gradient buffer-eluted fraction which had been derived from insoluble collagen t progressively raised temperatures contained in abundance imino acids and hydroxy amino acids (in comparison to soluble collagens

Denatured collagen can be divided into an arbitrary number of fractions using an Amberlite CG-50 column. eluted with buffers of increasing ionic strength and pH and finally with NaOH-solution (Kulonen *et al.* 1962) This method is suitable for the comparisons of such far-degraded gelatins. *e.g.* derived from insoluble collagens. which do not resolve to distinct fractions by *carboxymethylcellulose column chromatography* (Piez, Weiss and Lewis 1960, Kulonen. Virtanen and Salmenperä 1962) or by starch-gel electrophoresis (Näntö, Pikkarainen and Kulonen 1965)

The purpose of the present study was to apply this method to the comparison of collagens which differed in regard to the age and tissue of the animal or to the sol bility

Experimental

P eparation of various ollagens

The skins of growing (age one week) and full-grown (age unknown) cow and of growing (weight under 500 g) and full-grown weight 520—840 g, mean 650 g guinea pigs ere obtained immediately after killing The isolation and purification of neutral salt-soluble NSC

and acid-soluble (AC) skin collagens followed the principles of Gross, Highberger and Schmitt (1955) Gallop (1955) Gross (1958) and Pikkarainen and Kulonen (1968) The temperatures in Fig. 2—4 indicate (except 120°T) the insoluble collagen (IC) dissolved at progressively raised temperatures at 40 for 15 min, at 65 90 and 120 for 120 min (Pikkarainen and Kulonen 1968) The various fractions of insoluble collagen from the Achilles tendon of the calf were prepared in an analogous manner

Fractionation by Amberlite CG-50 column chromatography

The preparative column chromatography of gelatinized collagen by Amberlite CG-50 resin has been described in detail elsewhere (Pikkarainen and Kulonen 1968) Usually 3 fractions were pooled, the pH 5.5-buffer-eluted fraction, gradient-buffer-eluted fraction and NaOH-eluted fraction (occasionally divided into 0.1 N NaOH and 1.0 N NaOH-eluted fractions)

Analytical methods

The total *content of collagen* in the various Amberlite CG-50 fractions was obtained by an integration of the absorbance values obtained by a modified biuret reaction (Pikkarainen and Kulonen 1965) Standard curves used in these calculations were made for each fraction from an acid-processed pig-skin gelatin (Eastoe 1961) dissolved in 0.1 M Tris buffer (pH 5.50) in 0.5 M disodium phosphate solution, and in 0.1 N or 1.0 N NaOH.

For the determination of the *amino acid compositions* the samples were hydrolysed in 5.7 N hydrochloric acid under nitrogen in sealed tubes at +110 C for 20 h. The amino acid analyses were carried out by an amino acid analyser built according to Spackman, Stein and Moore (1958)

For the determination of the *hydroxyproline content* the samples were hydrolysed in 5.7 N hydrochloric acid at +130 C for 3 hrs. The colour reaction was carried out according to Woessner (Method II) (1961) The collagen content was calculated by multiplying the hydroxyproline content by 7.5 assuming 13.7 % of hydroxyproline in collagen.

Results

Comparison of soluble and insoluble collagens

Neutral salt-soluble and acid-soluble collagens differ from the insoluble collagen so far that the first fraction, eluted with the pH 5.5-buffer is larger than the same fraction from insoluble collagen (Fig. 1) This difference is compensated by the large 1.0 N NaOH-eluted fraction from insoluble collagen.

The comparison was extended to the amino acid compositions of the gradient eluted fractions both from soluble and insoluble collagens (Table I) If both amino acid values in the IC-columns deviate from the respective values in the NSC- and



Fig. 1 Comparison of soluble and insoluble collagens. The distribution of Amberlite CG-50 fractions from gelatinized (at +120 C for 120 min) neutral salt-soluble (NSC) acid-soluble (AC) and insoluble (IC) collagens of young guinea pig skin. The ordinate indicates the percentage from total collagen.

TABLE I. Comparison of the amino acid composition of the gradient-eluted fractions from soluble and insoluble collagens of growing guinea pig skin. The Amberlite CG-50 chromatography is explained in the experimental section. The amino acid composition is expressed in residues per 1000 residues. The 0.1 N NaOH-eluted fraction of insoluble collagen is presented for comparison. NSC neutral salt-soluble collagen, AC acid-soluble collagen, IC soluble collagen. The values for serine and threonine are not corrected for the decomposition occurring during the hydrolysis and chromatography

Amino acid	Gradient-eluted			NaOH-eluted
	NSC	AC	IC	IC
4-Hydroxyproline	96	100	111	113
Aspartic acid	49	45	48	51
Threonine	18	18	18	20
Serine	33	33	36	39
Glutamic acid	63	64	66	6
Proline	112	113	116	125
Glycine	354	352	344	350
Alanine	124	121	114	104
Valine	20	20	18	.5
Methionine	4	4	6	0.5
Isoleucine	10	11	10	11
Leucine	21	22	22	24
Tyrosine	3	2	5	0.5
Phenylalanine	11	13	12	12
droxylysine	4	3	3	0.5
	1	0	0	0
Lysine	22	23	24	16
Histidine	5	3	5	0
Arginine	50	51	42	54
Acidic residues	112	109	114	127
Basic residues	82	82	4	50
Excess of acidic residues	30	27	40	77
Hydroxyproline proline	0.86	0.88	0 96	0 90
Hydroxy amino acids	154	156	173	172
Total mino acids	208	213	227	238

AC-columns, the differences ma be typical for those regions in tropocollagen, which are involved in the maturation of soluble collagens to insoluble. Such deviations are observed in both mino acids (high in IC hydroxy amino acids high in IC suggestively in glutamic acid (high in IC and in arginine low in IC

Comparison on the ag f the animal

Insoluble collagens of guinea pig and cattle skins were studied. The effect of the age on the thermal stability is better manifested n cattle Fig ? At ~65 C there is very marked difference between calf and cow in th amount of the gelatinized com-

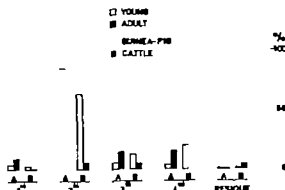


Fig. 2. Effect of the age of the animal on the thermal stability of insoluble ski collagen. The distribution of extracts obtained t stepwise heating at indicated temperatures is shown. The medium was 0.01 M acetate buffer pH 4.8. The ordinate indicates the percentage from total insoluble collagen.

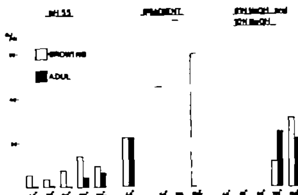


Fig. 3 The Amberlite CG-50 fractions from the various preparations of insoluble skin collagens of young and full-grown guinea pigs. The insoluble collagen (IC) was dissolved at progressively raised temperatures. 120 T means that IC was treated t 120 for 120 min without preliminary gelatinizations at lower tempera tures. The ordinate as in Fig. 1

ponents. This is reversed in the fraction which dissolves first t +120° C. Insoluble collagen of a growing animal yields relatively much of the pH 5.5-buffer-eluted fractions (Fig. 3) The NaOH-eluted fractions mirror the patterns of the pH 5.5-buffer-el ted fractions, except in the 120°T-series. During the aging insoluble collagen is so altered that after a thermal degradation it contains less of the pH 5.5-buffer eluted and more of the NaOH-eluted component.

There were no differences in th amino acid compositions of th pH 5.5-buffer el ted fractions of collagens from the calf and the cow

Comparison f the sk and tend n

The insoluble collagens of the skin and tendon were chosen to represent opposite examples of the arrangement of the collagenous fibres in the tissues the net like nd the parallel. Fig. 4 shows that there is a marked differenc between the kin and the tendon in the collagenous fraction which is obtained by heating at +40° C and el ted at pH 5.5 In the distribution of the Amberlite CG-50 fractions the denatured tendon collagen resembles the beha iour of collagen from young animals.



Fig. 4. Comparison of the insoluble collagens from skin and tendon. The Amberlite CG-50 fractions from the various preparations were obtained as explained in the legend of Fig. 3 and in the experimental section. The ordinate as in Fig. 1.

Discussion

The primary purpose of this work was to find from collagen soluble preparations where differences between the young and full-grown animals or between the soluble and insoluble collagens could be demonstrated. The pH 5.5-buffer-eluted and NaOH-eluted fractions obtained by the Amberlite CG-50 chromatography seem to provide suitable samples for such comparisons.

Collagen becomes less susceptible to thermal degradation at the formation of the tertiary structure, at the aging or at the formation of a network. Because the organization of collagen seems to cause a shift from pH 5.5-buffer-eluted fraction to NaOH-eluted fraction, it is relevant to ask which features in the amino acid composition are characteristic for both insoluble collagen and the NaOH-eluted fraction. There seems to be an agreement in the abundance of imino acids only (Pikkarainen and Kulonen 1968). The high content of hydroxyproline in both fractions from insoluble collagen provokes a question whether the hydroxylation of collagen proceeds during the stabilization of collagen. There are various degrees of hydroxylation of proline in collagens (Bornstein 1967a, b) from various tissues. Does the maturation affect those parts of the molecule which are rich in imino acids and probably contain an excess of acidic residues, of hydroxylated proline and of hydroxy amino acid in general. Studies on the evolution of collagen indicate that the content of imino acids has increased in higher animals, as also the thermal stability (Pikkarainen 1968).

It has been demonstrated repeatedly that the solubility of collagen decreases with advancing age: with rat-tail-tendon (Nageotte and Guyon 1934), with human skin (Bakerman 1962, Banfield 1952), with cattle and pig skin (Reich, Walther and Stather 1962), with rat skin (Mills and Bavetta 1966) and with rabbit skin (Nimni de Guia and Bavetta 1965). The increased number of cross-links and the prevalence of larger aggregates has also been demonstrated in older animals (Heikkinen and Kulonen 1964). The present work extends this concept to the dissolution at heating

which may be limited by similar factors as the solubility in the cold. At aging similar processes seem to continue inside the insoluble collagen, which cause its formation from the soluble precursor

The effect of age on that proportion of insoluble collagen which can be extracted at 40° already depends on pH and other conditions (Heikkinen, E., in preparation)

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References

BAKERMAN, S., Quantitative extraction of acid-soluble human skin collagen with age. *Nature* (Lond.) 1962, *196* 375—376.

BANFIELD, W G., The solubility and swelling of collagen in dilute acid with age variations in man. *Anat Rec* 1952, *114* 157—171

BORNSTEIN, P., The incomplete hydroxylation of individual prolyl residues in collagen. *J biol. Chem.* 1967a, *242* 2572—2574.

BORNSTEIN, P., Comparative sequence studies of rat skin and tendon collagen. I. E idence for incomplete hydroxylation of individual prolyl residues in the normal proteins. *Biochemistry* 1967b, *6* 3082 –3093

EASTOE, J E A semi-micro method for the determination of amino acids by ion-exchange chromatography *Biochem. J* 1961, *79* 652—656.

GALLOP P M., Studies on parent gelatin from ichthyocol. *Arch. Biochem.* 1955 *54* 501—512.

GROSS, J Studies on the formation of collagen. I. Properties and fractionation of neutral salt extracts of normal guinea pig connective tissue. *J exp. M d* 1958, *107* 247—265

GROSS, J J H. HIGHBERGER and F O SCHMITT Extraction of collagen from connecti tissue by neutral salt solutions. *P oc nat Ac d Sci.* (Wash) 1955, *41* 1—7

HEIKKINEN E. and E. KULONEN Age factor in the maturation of collagen. Intramolecular linkages in mildly denatured collagen. *Experientia* (Basel) 1964 *20* 310—312.

KULONEN, E., U K. VIRTANEN, J VILJANTO and P SEPPÄLÄ, Fractionation of gelatins using Amberlite CG-50 resin. *Acta chem scand* 1962, *16* 695—700.

K LONEN, E U K VIRTANEN and A. SALMENPERÄ, Fractionation of gelatins w th carboxymethylcellulose. *Act hem. scand* 1962, *16* 1579—1585

MILLS, B. G and L. A BAVETTA, Variation in extractable collagen of bone and skin w th age and growth. *J Geront* 1966 *21* 449—454

NAGEOTTE, J and L. GUYON Les propriétés physico-chemiques du collagène et leurs consequences morphologiques. *C R. Ass. Anat* 1934 29 408—433.

NIMNI, M. E E. DE GUI and L. A. BAVETTA, Changes in the quantity and nature of collagen in rabbit skin as function of age. *Nature* (Lond.) 1965 *207* 865—866

NÄNTÖ V J PIKKARAINEN and E. K LONEN Optimal conditions in the starch-gel lectrophoresis of heat-denatured collagen. *J Amer Leather Chem Ass.* 1965 60 63—71

PIEZ, K A E. WEISS and M S LEWIS, The separation and characterization of the α- and β-components of calf skin collagen. *J biol Chem.* 1960 *235* 1987—1991

PIKKARAINEN J and E. KULONEN The fractionation of various gelatin preparations with ion-exchange resins. *Suomen Kemistilehti B* 1965 *38* 226—230

PIKKARAINEN, J The molecular structures of ertebrate skin collagens. A comparative study *Act physiol scand* 1968, Suppl. 309.

PIKKARAINEN J and E. KULONEN, The composition of the products of the Amberlite CG-50 fractionation of denatured collagen. *Act hem. scand* 1968 In press

REICH, G S WALTHER and F S ATHER, Untersuchungen über Kollagen und Gelatine IV Über den Einfluss des Alters on Rinds- und Schweinehäuten auf Ausbeut und Eigenschaf ten daraus nach dem sauren Aufschlussverfahren gewonnener Gelatine *Ges. Abh DLI* 1967 *18* 24—30.

SPACKMAN, D H W H. STEIN and S. MOORE, Automatic recording apparatus for use in the chromatography of amino acids *Analyt Chem.* 1958, *30* 1190—1206.

WOESSNER, J J F The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. *A h Biochem* 1961 *93* 440—447

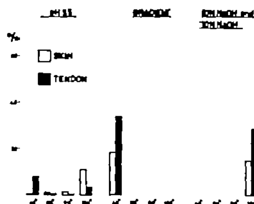


Fig. 4. Comparison of the insoluble collagens from skin and tendon. The Amberlite CG-50 fractions from the various preparations were obtained as explained in the legend of Fig. 3 and in the experimental section. The ordinate as in Fig. 1

Discussion

The primary purpose of this work was to find from collagen soluble preparations where differences between the young and full-grown animals or between the soluble and insoluble collagens could be demonstrated. The pH 5.5-buffer-eluted and NaOH-eluted fractions obtained by the Amberlite CG-50 chromatography seem to provide suitable samples for such comparisons.

Collagen becomes less susceptible to thermal degradation at the formation of the quaternary structure, at the aging or at the formation of a network. Because the organization of collagen seems to cause a shift from pH 5.5-buffer-eluted fraction to NaOH-eluted fraction, it is relevant to ask which features in the amino acid composition are characteristic for both insoluble collagen and the NaOH-eluted fraction. There seems to be an agreement in the abundance of imino acids only (Pikkarainen and Kulonen 1968). The high content of hydroxyproline in both fractions from insoluble collagen provokes a question whether the hydroxylation of collagen proceeds during the stabilization of collagen. There are various degrees of hydroxylation of proline in collagens (Bornstein 1967a, b) from various tissues. Does the maturation affect those parts of the molecule which are rich in imino acids and probably contain an excess of acidic residues, of hydroxylated proline and of hydroxy amino acids in general. Studies on the evolution of collagen indicate that the content of imino acids has increased in higher animals, as also the thermal stability (Pikkarainen 1968).

It has been demonstrated repeatedly that the solubility of collagen decreases with advancing age: with rat tail tendon (Nageotte and Guyon 1934), with human skin (Bakerman 1962, Banfield 1952), with cattle and pig skin (Resch, Walther and Stather 1962), with rat skin (Mills and Bavetta 1966) and with rabbit skin (Nimni, de Guia and Bavetta 1965). The increased number of cross-links and the prevalence of larger aggregates has also been demonstrated in older animals (Heikkinen and Kulonen 1964). The present work extends this concept to the dissolution at heating

leaving the static system to be dealt with in a following paper The dynamic fusimotor fibres influence almost exclusively the primary endings of the muscle spindles (Appelberg, Bessou and Laporte 1966) On activation dynamic fibres cause a prominent increase in the dynamic component of the response of these endings to stretch (Matthews 1962 Crowe and Matthews 1964a b)

Results obtained on passive endings (Lennerstrand 1968) will serve as a background for the papers in which the effects of fusimotor fibre stimulation are reported. In the study of the non-activated endings it was found useful to analyze the responses to length changes in terms of the different parameter which are indicated in the schematic impulse frequency—muscle length diagram of Fig 1 *B*. In addition to the components of the response due to the steady discharge at zero extension and the position sensitivity the response was considered as composed of two additive velocity components, which developed along quite different time courses (Fig. 1 *A*) They were denoted the quick and the 'slow' component respectively The quick component was regarded as a 'proportional' response following the input velocity signal. It can be characterized by its amplitude and its time constant. The latter however was hard to estimate with any accuracy and, therefore, only the amplitude was measured (Fig 1 *B*) Because of its long rise time, the slow component may be thought of as an 'intergral' of the velocity signal superimposed on the quick component. It was given the dimensions of a slope (Fig 1 *B*) In the present study

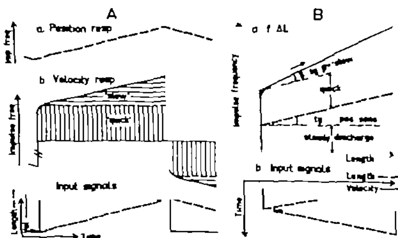


Fig 1 *A*. Schematic presentation on time scale of the impulse frequency response of spindle ending to length changes of triangular wave form. The total response is regarded as composed of ( ) length dependent and (*b*) elocity dependent part, The eloc ty response in turn, is divided into two components, quick and 'slow' one of different time ou ses The input signals to yield the responses in and *b* are shown in

B. The total response in an impulse frequency (*f*)—muscle length (ΔL) plot Res length increment only fully represented, as indicated by the input gnals *b* developed in time as shown by arrow in The length and the elocity depende add to gi the total response. The graphical analysis of the *f*-ΔL curv and the on the different parameters shown in *a*. (The steady discharge at 'zero the starting point of the *f* ΔL response t length increment) Note by the length signal, the abscissa of the plot is linear with respect both t

the influence on these four parameters of dynamic fusimotor activation has been determined.

Some of the results have been reported in a preliminary note (Lennerstrand and Thoden 1967).

Methods

The general procedures and the experimental technique have already been fully described (Lennerstrand and Thoden 1968). Spindle endings could be identified from the conduction velocity of their afferent fibres and from their responses in the f-ΔL diagrams to triangular extensions (*cf.* Lennerstrand 1968). All endings, identified from f-ΔL diagrams as belonging to the so-called intermediate type (*cf.* Lennerstrand 1968), have been included in the present material on primary endings.

By the method of isolating single fusimotor fibres used in this study, fibres of the so-called β-type (Adal and Barker 1965) have probably been excluded. The notion is of relevance in this connection since the fusimotor branch of the β-fibres has been reported to be of the dynamic type. Qualitatively the effects of stimulating β-fibres do not differ from those induced by dynamic fusimotor fibres of γ-type (Bessou, Emonet-Dénand and Laporte 1965; Brown, Crowe and Matthews 1965). However, since the effects might turn out to be different if tested with the present technique, the results of this study cannot immediately be regarded as representative for all dynamic fibres. The material comprises 31 dynamic fusimotor fibres destined for extensor spindles and 6 for flexor spindles. The conduction velocities of the fibres ranged between 19 and 45 m/sec. However, the number of dynamic fusimotor fibres studied with relatively complete dynamic analysis was only about one third of the total number, since many intrafusal fibres did not resist the strain of repeated tetanic stimulation. In two instances a primary ending was found to be innervated by two different dynamic fusimotor fibres. No investigation was made of multiple ending innervation by single fusimotor fibres.

Results

Steady discharge and position sensitivity

In response to steady dynamic fusimotor fibre stimulation the impulse frequency of a primary ending rose slowly in less than one second up to a constant value. Overshoot in the initial part of the response was never seen (*cf.* Crowe and Matthews 1964*b*). The steady state values during fusimotor activation were determined in the second half-second of constant stimulation in order to avoid the influence of intrafusal fatigue. In the early part of this investigation it was thought that complete spindle adaptation to a rapid change of length was necessary before an accurate measurement of the steady firing rate during fusimotor activation could be obtained. Thus the sequence of events was: rapid muscle length change—30 sec interval—and begin fusimotor fibre stimulation. It was soon found out, however, that this time interval could be shortened to 2 sec without influencing the steady state value. This advantage of saving experimental time had to be balanced against the disadvantage of exhausting the intrafusal fibre by too frequently applied stimulation. Accordingly the endings were permitted to rest for 10—15 sec between activations.

As observed by others, the steady discharge rate or the position response of the endings to zero extension (*cf.* Fig. 1B) was only moderately increased by dynamic fusimotor activation. This is in contradistinction to the effects of static fusimotor fibres, which usually gave rise to a marked increase in the position response (Matthews 1962; Appelberg, Bessou and Laporte 1966). At the rate of dynamic

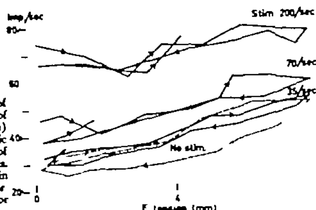


Fig. 2. Steady state f-ΔL curves of primary ending deprived of fusimotor activation (dashed line) and during constant dynamic fibre stimulation (full lines) of the rates marked at the curves. The arrows show the direction in which the values are obtained i.e. in sequence of increasing or decreasing lengths.

fibre stimulation of 70 imp/sec the increase in position response in our material was 25 ± 11 S.D. imp/sec (17 observations).

In Fig. 2 steady state f-ΔL curves of a primary ending have been plotted in the absence of fusimotor activation and during stimulation of a single dynamic fusimotor fibre. The increase in position response is seen to be very small at a rate of stimulation of 35/sec, although a considerable increase in dynamic sensitivity was observed. In this particular ending the hysteresis of the steady state curve was reduced by the dynamic fusimotor activation. Usually there were no changes in the hysteresis to be observed. The position sensitivity defined in Fig. 1B is typically seen to be lowered as a result of the dynamic fusimotor activation. The position sensitivity of the non-activated ending is 3.4 imp/sec/mm. At the rate of stimulation of 35/sec and 70/sec the slope has decreased to 3.1 imp/sec/mm and at 200/sec the slope value is about 2 imp/sec/mm. In other endings the position sensitivity was not changed by the dynamic fibre stimulation. Average values for the change in position sensitivity of extensor primary endings at different rates of dynamic fusimotor stimulation are given in Table I. Too few observations have been made on flexor spindles to merit calculations of mean values.

Velocity sensitivity

As described in a previous paper (Lennerstrand 1968) the velocity sensitivity of a spindle ending has been determined from the relations between input velocity and

TABLE I. The change in position sensitivity of extensor primary endings induced by dynamic fusimotor fibre stimulation. Mean values and standard deviations given in imp/sec/mm. Rates of fusimotor stimulation in shocks/sec.

Rate of stimulation	Mean ± S.D	
35	−0.2	5
70	−0.8 ± 1.1	17
200	−1.8 ± 1.6	16

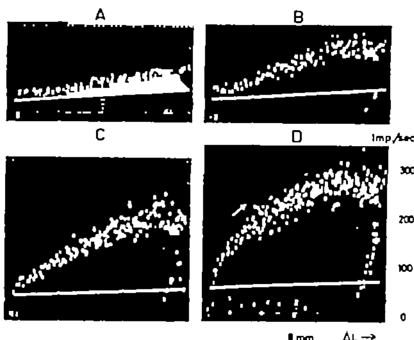


FIG. 3 f ΔL diagrams of primary ending to 8 mm/sec 'triangular' changes of length. A no fusimotor stimulation. B during dynamic fibre stimulation at 33 pulses/sec C at 70 pulses/sec and D at 200 pulses/sec Solid lines are steady state curves approximated to straight lines Arrow shows the point movement of the plots in time Length (ΔL) increases to the right in the picture

velocity responses when applying 'triangular' length changes. The subdivision of the velocity response into a 'quick' and a 'slow' component (see Fig 1) proved to be valid also when the primary endings were under dynamic fusimotor control. This is recognized from Figure 3 which shows the typical f ΔL diagrams of a primary ending in the non-activated state (A) and during dynamic fusimotor activation (B C and D) In all these records the muscle was subjected to length changes of 8 mm sec. In Fig. 4 the same ending is shown in the non-activated state (A) and during dynamic fibre stimulation (B) at 70 pulses sec but at the lower velocity of 2 mm sec

'Quick' and 'slow' components have been assessed as shown in Fig. 1 B. Before describing the dynamic fusimotor effects on the velocity components, some features particular for the responses during dynamic activation warrants mentioning Measurements of the velocity response could be performed only in the response to the length increments, since the primary endings ceased firing early in the phase of muscle shortening also when activated by dynamic fusimotor stimulation. Velocity responses to length decrements will be treated later in a separate section. At high rate stimulation the 'quick' component usually had a slightly longer time constant than in the non-activated state (see Fig 3 A and D) However attempts to estimate the time characteristics of the 'quick' responses during dynamic activation have failed the time course varied with the velocity of length change at a defined rat

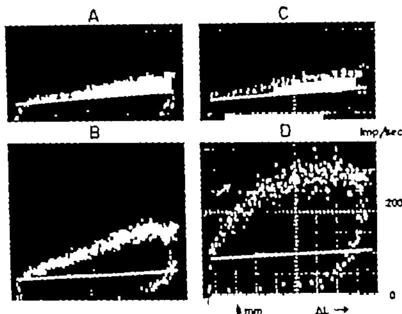


Fig. 4 *A* and *B*, *f* ΔL diagrams of the same ending as in Fig. 3 but t triangular length input of 2 mm/sec. *A* non-activated rate. *B* during dynamic fibre stimulation of 70 pulses/sec. *C* and *D* *f* ΔL diagrams of the same ending but at 2.5 mm increased initial extension 8 mm/sec rate of length hange *C* non-activated, *D* dynamic activation t 200 shocks/sec Markings as in Fig. 2.

of stimulation During relatively strong dynamic fusimotor act ation the impulse frequency curve to length increment often levelled off at the largest extensions (Fig. 3 *D*) This part of the response will also be dealt with separately n a later section. Initial burst responses (*cf* Lennerstrand and Thoden 1968, also for references) were sometimes observed during low rate dynamic stimulation (Fig 3 *B*) but they were usually absent during stimulation at higher rates. For reasons already discussed (*cf* Lennerstrand and Thoden 1968) they are disregarded in the quanti tative description of the velocity response

Q ick velocity espons to length increment This elocity response increased with the rate of fusimotor stimulation and the elocity of stretch In the graph of Fig 5 *A* the quick velocity responses of the ending in Fig 3 and 4 ha been plotted against velocity The relation between quick component and elocity which for the non-activated ending was almost linear became more exponenti l d ring the dynamic fusimotor activation

The increase in the quick response at dynamic fibre t m l tr bov the l of the non-activated ending is in Fig 6 illustrated for l whole popul t n l endings. Each curv represents values of one ending In t ases th ncre in quick response with input elocity was smaller n the a t ted than the pas ive ending as indicated by the negative slopes of some of tl urves in th pl ts of Fig. 6 *A* and *B* The filled circles represent mean al es f tensor nd g No

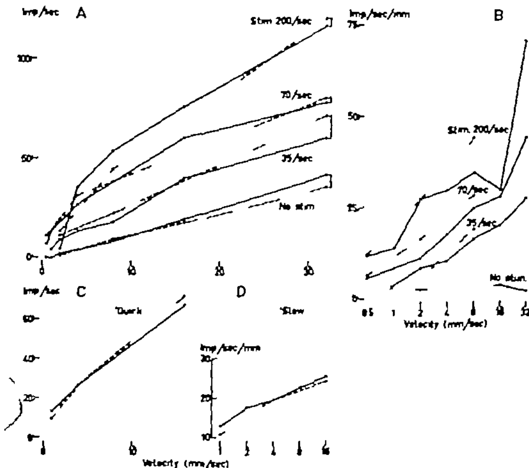


Fig 5 *A* Plots against elocity of the quick velocity esponses of primary ending of F g. 3 and 4 in the absence and the presence of dynamic fusimotor acti tion Rates of stimulation as marked at curves Broken lines represent alues obtained when initial muscle extension was increased 2 5 mm. Control valus are marked by full lines. *B*. 'Slow velocity responses of the same ending gainst log alues of locity Full and broken lines as in *A* *C* 'Quick velocit responses of another primary ending during dynamic fibre stimulation of 70 pulses/sec Solid line fitted to alues from 'triangular movements of 8 mm amplitude and broken line from 4 mm amplitude round the same operating point *D* 'Slow elocity responses of the ending in C and oder he same experimental conditions

differences were obtained between quick responses of endings in soleus or the lateral gastrocnemius muscles. 'Quick velocity responses of flexor endings (interrupted lines) fall within the range of responses of extensor endings (full lines)

Slow' velocity ponse t length m ment It is clear from Fig 3 and 4 that in primary endings the most prominent effect of dynamic fusimotor activation was the large increase in the slope of the dynamic *f* lL diagrams. i.e in the slow component of the velocity response The slow responses of the ending of F'g 3 and 4 at different rates of stimulation are plotted in Fig. 5 *B* against log alues of velocity The curves in the plot r roughly rectilinea The curves to fit the mean v lues

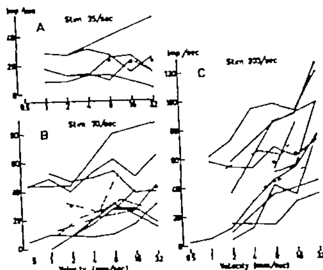


Fig. 6. The increase in quick velocity response (above the value of the passive ending) caused by dynamic fusimotor fibre stimulation. Each line represents the effects of one dynamic fibre on one primary ending. Velocity on log scale. Solid lines mark extensor endings and broken lines flexor endings. Filled circles are average values for the population of extensor endings. *A*, *B* and *C* differ with respect to rate of fusimotor fibre stimulation.

against log velocity of the increase in slow response (above the value in the non-activated state) by dynamic fusimotor activation also fall on approximately straight lines (Fig. 7).

A *couple of flexor endings (interrupted lines) are also represented in* Fig. 7. During dynamic fusimotor activation some of them had slow responses larger than those of extensor endings. These particular endings were all located in the peroneus muscles. The reason for their having the largest 'slow' response is probably that, because this muscle is fairly short, they have been subjected to relatively larger extensions than endings of other muscles (Lennerstrand 1968).

'Frequency ceiling'. During stimulation of dynamic fibres, a levelling off was often seen in the impulse frequency responses to length increments at the highest extensions (Fig. 3 *C* and *D*, Fig. 4 *D*). This has been observed earlier by Harvey and Matthews (1961) in primary endings activated by stimulation of single fusimotor fibres, which, however, were unidentified with respect to dynamic and static fusimotor effects. The impulse frequency at which the levelling off occurred, increased with increasing velocity of movement and increasing rate of fusimotor stimulation (*cf.* Fig. 8 *B*). The phenomenon can therefore not be considered as a saturation of the response in the usual sense of the word. It seems also improbable that the levelling off depended on intrafusal fibre fatigue, since the same level was reached in several consecutive cycles of triangular length changes during constant fusimotor fibre stimulation (see Fig. 4 *D*).

The term 'frequency ceiling' has been used to describe this behaviour. It is mean-

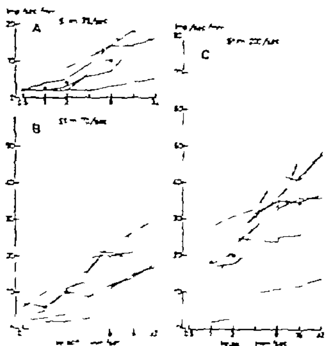


Fig. The increase in slow velocity response by dynamic fusimotor activation presented in the same way as in Fig. 6 and with the same labelling.

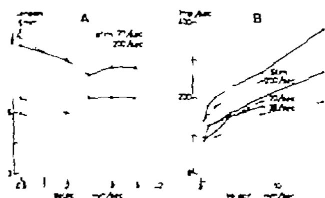


Fig. 8. Frequency ceiling of the ending in Fig. 3 and 4. A Plot against log velocity of the amount of extension at which frequency ceiling first appeared in the f ΔL curves. The rates of stimulation of 0 and 200 pulses/sec are represented. Full lines indicate values at an original initial muscle extension and broken lines represent values at 2.5 mm increased initial extension. Note change in initial extension induces shift to lower extensions in the starting point of the frequency ceiling. The shift corresponds approximately to the change in initial extension. B Plot against log velocity of the level of frequency ceiling. Rates of stimulation marked the curves. Solid and broken lines indicate the same shifts in initial extension as in A

ured as the impulse rate at levelling off. Sometimes the f ΔL diagrams, instead of a level, showed a summit point followed by a decline as the stretch proceeded. In these cases the top impulse rate has been taken as the frequency ceiling.

The average values of frequency ceiling for samples of extensor and flexor endings were distributed along almost straight lines in log log plot against velocity (Fig. 9 A and B). The values of flexor endings in Fig. 9 (broken lines) fall within the range of values for extensor endings.

The muscle extension at which levelling off first occurred has been plotted

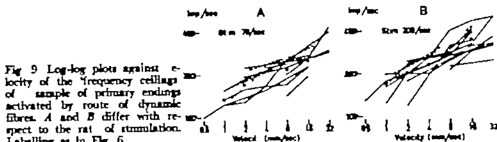


Fig 9 Log-log plots against velocity of the 'frequency ceilings' of sample of primary endings activated by route of dynamic fibres. *A* and *B* differ with respect to the rat of stimulation. Labelling as in Fig. 6.

Fig 8 *A* for the ending of Fig 3 and 4 The value of this extension is seen to decrease with increasing input velocity Usually the levelling off started at lower extensions with increasing rate of fusimotor fibre stimulation, as observed in the ending plotted in Fig 8 *A*

Variations in initial tension and amplitude of the length change Responses to triangular length changes of increased initial muscle length or of reduced amplitude were also recorded, in order to find out whether shifts in the operating point and or in the operating length range of the endings possibly influenced their velocity sensitivity during dynamic fusimotor fibre stimulation.

No changes were found by these procedures in the quick and the slow' velocity responses to length increment or in 'frequency ceiling Fig. 4 *C* and *D* illustrates the results in *f* ΔL diagrams of subjecting the ending of Fig 3 to length changes at 2.5 mm increased muscle length. The velocity responses thereby obtained have been plotted in Fig 5 *A* and *B*. Fig. 5 *C* and *D* illustrate another primary ending. *The full lines connect the velocity responses obtained in triangular length changes* with an amplitude of 8 mm. The broken lines represent th responses to length changes around the same operating point but of 4 mm amplitude. The 'frequency ceilings' of the ending in Fig 3 and 4 to length changes of d fferent initial extension are plotted in Fig. 8 *B*.

However the extension at which the levelling off of the responses started could be shifted to lower alues by increasing the initial muscle length. This is shown in Fig. 8 *A*

Responses during el ase f stret h In the non-acti ated tate most primary endings ceased firing immediately on reversing the direction of the muscl length change from elongation to shortening, when the numerical alue of eloc ty exceeded 0.5 mm sec (Lennerstrand 1968) By this beha iour measurements n velocity responses during release of stretch were rendered impossible.

On dynamic fusimotor activation the impulse f ring to length decrement was maintained longer than in the non-activated state (see F g nd 4) but normall the impulse frequency representation of the phase of muscle shortening in th *f* ΔL diagrams was insufficient for exact measurements on the elocity response. The impulse firing to length decrement could be increased further in duration by increasing the rate of stimulation and augmenting the initial extension of th muscle

(Fig. 4 *D*). Then the two transients signifying the 'quick' and the 'slow' velocity response could be determined. They were found to be similar in time course to those obtained in muscle lengthening.

Some primary endings fired impulses at all parts of length decrease. In these cases the impulse frequency had a constant value during the later part of the muscle release as also shown by Crowe and Matthews (1964*b*). Under such circumstances, the total velocity response to length decrement could be determined. It was found to be much smaller than that to length increment. In Fig. 4 *D* this can be seen by comparing the areas above or below the steady state curve circumscribed by the *f*–ΔL diagram. Since in no case the magnitude of the corresponding velocity responses to muscle shortening of the non-activated primary ending could be exactly determined, it is unknown whether the total velocity response to length decrement was changed or not by the dynamic fibre stimulation.

Endings of intermediate type. In the non-activated state the endings of this type had response characteristics of both primary and secondary endings (*cf.* Lennerstrand 1968). In 6 cases responses have been recorded from 'intermediate' endings during dynamic fusimotor activation. They were indistinguishable from other primary endings activated by dynamic fibre stimulation with respect both to the general shape of the *f*–ΔL curves and the magnitude or the relation to velocity of quick and 'slow' velocity responses to length increment. Usually the 'intermediate' endings showed a mean frequency of impulse firing at length decrement during stimulation that ordinary primary endings reached only at increased initial extension, but only in this respect did the responses to length decrement of the 'intermediate' endings differ from those of ordinary primary endings.

Discussion

Constant dynamic fusimotor stimulation has in the present paper been found to influence both the static and the dynamic properties of primary muscle spindle endings to length changes. Using the results obtained in the non-activated endings as controls, the dynamic activation was seen to 1) increase rather moderately the steady discharge rate to zero muscle length, as has been reported earlier by Crowe and Matthews (1964*a*), 2) lower the position sensitivity and 3) increase the velocity sensitivity markedly as was also shown by Crowe and Matthews. In its effects on the velocity sensitivity the dynamic activation was found to induce a relatively larger increase in the 'slow' component than the quick component. Moreover the range of velocities within which the 'slow' component was responsive was expanded during dynamic fibre stimulation; the range became about the same as that for non-activated secondary endings (*cf.* Lennerstrand 1968).

In the non-activated state the time constant of the quick component was very short, but during dynamic activation some increase with the rate of stimulation was seen. The quick component during strong activation may well correspond to one of the components observed by Crowe and Matthews (1964*a*) in the decay of the discharge on completion of stretching during dynamic fusimotor activation. The time constant of the slowest component of the decay was by these authors estimated to about 0.5 sec. This value, however, is too low to account for the slow velocity response found in the present work.

The extensive discussion on the significance of the reported dynamic fusimotor effects on primary endings from the point of view of muscle length control will be postponed until the results of the analogue spindle simulation have been presented It can be briefly mentioned, however that the 'slow velocity response by its time course serves to integrate the response to the dynamic part of the length change (see also Crowe and Matthews 1964a) the 'slow component can be regarded as a velocity dependent gain of the response. The gain factor is increased by dynamic fusimotor activation. Velocity sensitivity in the usual sense of the word would then be signalled by the quick velocity response. Also this component is increased by the dynamic fusimotor stimulation. Furthermore the results obtained during intensive dynamic fibre stimulation indicate that the primary endings became more or less undirectionally sensitive to elocity the velocity response to length decrement dur ing dynamic fusimotor activation was much smaller than the response to length increment. The significance of this finding will also be discussed in connection with the results of the analogue spindle simulation.

Regarding the structural correlate to the spindle beha our during dynamic fusimotor activation, a body of evidence exists for the opinion that the responses derive from sensory endings on nuclear bag fibres. This is a point of importance with respect not only to the mechanical explanation of dynamic fusimotor effects but also to the interpretation of the responses in $f/\Delta L$ diagrams obtained on stimulating two isolated fusimotor fibres, one or both of them dynamic, converging on the same ending One piece of evidence for this is the physiological finding that dynamic fusimotor fibres in the cat almost exclusively influence only the primary endings, which always have at least one receptor site in a nuclear bag fibre, whil secondary endings ery rarely innervate these intrafusal fibres.

For the further argumentation a comparison with amphibian muscle physiology is needed. By their contractional properties observed directly under the microscope the nuclear bag fibres have been equated with 'slow muscle fibres of the frog (Boyd 1966a b Diete-Spiff 1966, Smith 1966) while nuclear chain fibres bear more resemblance to twitch fibres (Boyd 1966a, b Smith 1966) In analogy to the electrical properties of the slow muscle fibres, local action potentials ha e been recorded from the intrafusal muscle on stimulation of dynamic fusimotor fibres in the cat (Bessou and Laporte 1965) Furthermor steady stimulation of small motor fibres in the amphibians innervating slow intrafusal fibres (Eyzaguirre 1957 1958) elicited dynamic fusimotor effects to stretch in the frog pindle (M tthew and Westbury 1965) Thus, dynamic fusimotor effects seem to depend on excitation of spindle elements with properties of slow muscle fibres, i.e nuclear bag fibres.

Another piece of evidence has been supplied by Rack and Westbury (1966) They found that the well-known activating effect on the spindl f succinylcholine (Granit, Skoglund and Thesleff 1953 for further references see M tthews 1964) a com petitiv acetylcholine inhibitor elicited dynamic fusimotor responses to stretch in cat primary endings at a time after injection when contraction of all but slow muscle fibres must have subsided motor end plates on slow fibres are known to be much

more resistant to blocking by anti-cholinergic drugs than the endplates on twitch fibres (*cf* Kuffler and Vaughan Williams 1953) Direct microscopical observations on isolated spindles have also shown that the contraction of nuclear bag fibres to succinylcholine was stronger and more prolonged than that of nuclear chain fibres (Smith 1966)

In this connection it also warrants mentioning that the tension changes induced by stretching activated slow muscle fibres of the toad (Lännergren, personal communication) have been found to resemble the impulse frequency changes of dynamic fusimotor type obtained in response to stretch of cat primary endings. Moreover a tendency to lower the slope of the steady state length tension relation with increasing degree of muscle fibre activation is definitely not a characteristic of twitch fibre mechanics (Buchthal and Kaiser 1944 Frank 1965) Possibly a first step towards understanding the mechanical properties of nuclear bag fibres would be by analyzing the length-tension relations in isolated slow fibres of amphibians.

According to the present understanding of the efferent innervation of mammalian muscle spindles reviewed by Barker (1967) not all fusimotor fibres to nuclear bag fibres are of the same type This probably means that not all of them are dynamic fibres in function. Support to this view is given by results from physiological st idies on rabbit spindles. The rabbit spindle contains no nuclear chain fibres but only nuclear bag fibres (Barker and Hunt 1964) Nevertheless, Emonet Dénand, Laporte and Pagès (1966) found that single fusimotor fibres could be isolated in the rabbit which on stimulation gave rise to either dynamic or static effects. From these findings it has been assumed that also the nuclear bag fibres of the cat would be able to [illegible]nerate responses of both dynamic and static type (Appelberg Bessou and Laporte [illegible]966) but this remains to be shown physiologically

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References

Ad[illegible], M [illegible] and D Barker Intramuscular branching of fusimotor fibres. *J Physiol.* Lond 1965 *177* 288—299

A[illegible]lberg, B P Be[illegible]o[illegible] and Y Laporte Action of static and dynamic fusimotor fibres on secondary endings of cat spindles. *J Physiol* (Lond) 1966 *185* 160—171

Barker D The innerv[illegible]tion of mammalian skeletal muscle I[illegible] *C[illegible] Fo[illegible] Symposium on Myotatic [illegible]* Eds A V S de Reuck and J Knight, Churchill, London 1967 pp 3 15

Barker, D and J P H[illegible] Mammalian intrafusal muscle fibres. N[illegible] (Lond) 1964 *203* 1193

Bessou P F Emon[illegible] D[illegible] and Y Laporte Motor fibres innervating extraf[illegible] and intrafusal muscle fibres [illegible] the cat *J Physiol* (Lond.) 1965 *180* 649—672

Be[illegible] P and Y L[illegible] Potentiels fusoriaux provoqués par la stimulation de fibres [illegible]motorices chez le Cha[illegible] *C[illegible] S[illegible]* Paris 1965 *260* 4827—4830

Boyd, I A., The behaviour of isola ed mammalian muscle spindles w th intact [illegible] *J Physiol.* (Lond.) 1966a *186* 109 110 P

Boyd I A., The mechanic l properties of mammalian intrafusal muscle fibres. *J Physiol.* (Lond.) 1966b *187* 10- 1 P

Brown M C., A. Crowe and P B C M[illegible] Observ tion on the fusimotor fibres of the tibialis posterior muscle of the [illegible] *J Physiol* (Lond. 1965 *177* 140—159

BUCHTHAL, F. and E. KAISER, Factors determining tension development in skeletal muscle. *Acta physiol. scand.* 1944. *8* 38—74.

CROWE, A. and P. B. C. MATTHEWS, The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles. *J. Physiol.* (Lond.) 1964a. *174* 109—131.

CROWE, A. and P. B. C. MATTHEWS, Further studies of static and dynamic fusimotor fibres. *J. Physiol.* (Lond.) 1964b. *174* 132—151.

DIETE-SPIFF, K., Time course of mammalian intrafusal muscle contraction as revealed by cinephotography of isolated lumbrical muscle spindles of the dog. *Arch. ital. Biol.* 1966. *04* 387—405.

EMONET-DÉNAND, F., Y. LAPORTE and B. PAGÈS, Fibres fusimotorices statiques et fibres fusimotrices dynamiques, chez le Lapin. *Arch. ital. Biol.* 1966. *104* 195—213.

EYZAGUIRRE, C., Functional organization of neuromuscular spindle in the toad. *J. Neurophysiol.* 1957. *20* 523—542.

EYZAGUIRRE, C., Modulation of sensory discharges by efferent spindle excitation. *J. Neurophysiol.* 1958. *1* 465—480.

FRANK, G. B., Definitions of two distinct processes in activation during potassium-induced contractures of frog skeletal muscle. *Acta physiol. scand.* 1963. *63* 1—10.

GRANIT, R., S. SKOGLUND and S. THESLEFF, Activation of muscle spindles by succinylcholine and decamethonium. The effect of curare. *Acta physiol. scand.* 1953. *28* 134—151.

HARVEY, R. J. and P. B. C. MATTHEWS, The response of de-efferented muscle spindle endings in the cat's soleus to slow extension of the muscle. *J. Physiol.* (Lond.) 1961. *157* 370—392.

JANSEN, J. K. S. and P. B. C. MATTHEWS, The central control of the dynamic response of muscle spindle receptors. *J. Physiol.* (Lond.) 1962. *161* 357—378.

KUFFLER, S. W. and E. M. VAUGHAN WILLIAMS, Properties of the slow skeletal muscle fibres of the frog. *J. Physiol.* (Lond.) 1953. *121* 318—340.

LENNERSTRAND, G., Position and velocity sensitivity of muscle spindles in the cat. I. Primary and secondary endings deprived of fusimotor activation. *Acta physiol. scand.* 1968. *73* 281—299.

LENNERSTRAND, G. and U. THODEN, Fusimotor effects on position and velocity sensitivity of muscle spindles. *Experientia* (Basel) 1967. *23* 205—206.

LENNERSTRAND, G. and U. THODEN, Dynamic analysis of muscle spindle endings in the cat using length changes of different length-time relations. *Acta physiol. scand.* 1968. *73* 234—250.

MATTHEWS, P. B. C., The differentiation of two types of fusimotor fibre by their effects on the dynamic response of muscle spindle primary endings. *Quart. J. exp. Physiol.* 1962. *47* 324—333.

MATTHEWS, P. B. C., Muscle spindles and their motor control. *Physiol. Rev.* 1964. *44* 219—2[illegible].

MATTHEWS, P. B. C. and D. R. WESTBURY, Some effects of fast and slow motor fibres on muscle spindles of the frog. *J. Physiol.* (Lond.) 1965. *178* 178—192.

RACK, P. M. H. and D. R. WESTBURY, The effects of suxamethonium and acetylcholine on the behaviour of cat muscle spindles during dynamic stretching and during fusimotor stimulation. *J. Physiol.* (Lond.) 1966. *186* 698—713.

SMITH, R. S., Properties of intrafusal muscle fibres. In *Nobel Symposium I. Muscular Afferents and Motor Control*. Ed. R. Granit. Stockholm 1966. Almqvist and Wiksell. pp. 69—80.

Acta physiol. scand. 1968. 74. 30—49

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Position and Velocity Sensitivity of Muscle Spindles in the Cat. III. Static Fusimotor Single-Fibre Activation of Primary and Secondary Endings

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Abstract

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During the tetanic stimulation of single static fusimotor fibres, primary and secondary endings in ankle extensor and flexor muscles of the cat have been subjected to triangular length changes. Position and velocity sensitivities were determined and compared with those obtained in non-activated endings. Two types of action of static fibre stimulation on the position sensitivity have been observed both in primary and secondary endings. It was either unchanged or it decreased with increasing rate of stimulation (type I effect) or it increased with increasing rate of stimulation (type II effect). The distribution of the two types was different to primary and secondary endings and to extensor and flexor endings. During stimulation of most static fibres, irrespective of their type, the velocity responses of both primary and secondary endings resembled those of non-activated secondary endings. The 'quick' component of the velocity response was usually larger in primary than in secondary endings, while the slow components were about the same. Comparisons made of secondary endings between the velocity responses during static activation and in the non-activated state showed that the 'quick' component was reduced and the slow component increased by the stimulation. 'Exceptional' velocity responses were observed in type I activation of some primary endings.

With the definition given by Jansen and Matthews (1962*a*) and Matthews (1962) static fusimotor fibres exert an effect of diminishing the dynamic response of spindle endings to stretch. The present paper, which is a continuation of an extensive investigation with the purpose of describing quantitatively the static and the dynamic properties of spindle endings, deals with the influence of the static fusimotor system on primary and secondary endings. In preceding papers the responses to length changes have been regarded as consisting of a position and a velocity response component. The latter could be further divided into a 'quick' and a 'slow' component

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(Lennerstrand 1968) Dynamic fusimotor activation was found to enhance the slow component more than the quick one (Lennerstrand and Thoden 1968b) It remains unknown, however in which way static fibre stimulation effects the two components of the velocity response Neither has the static fusimotor action on the position response been systematically explored.

Static fibres are known to influence the discharge pattern of both primary and secondary endings (Appelberg, Bessou and Laporte 1966 Brown and Matthews 1966) Recent studies of spindle efferent innervation (cf Barker 1967) strongly suggest that in primary endings both the nuclear bag and the nuclear chain components may contribute to static fusimotor responses. A study employing the present methods might shed some light on the problems concerning the mechanisms underlying the response effects, and on the question whether static fusimotor actions can be mediated by both types of intrafusal muscle fibre

The comparison between responses of endings in flexor and extensor muscles started in earlier parts of this study has been pursued also during static activation, with the hope of revealing functional differences between spindles in the two muscle groups.

Parts of the results have been reported earlier in a preliminary work (Lennerstrand and Thoden 1967)

Methods

The general procedures and the technique have been fully described in previous paper (Lennerstrand and Thoden 1968a) The number of endings studied with full dynamic analysis was smaller than that on which the position sensitivity was determined, since all spindle endings did not resist the strain of being repeatedly stimulated as often as was necessary for the dynamic analysis.

The functional identification of static fusimotor fibres followed the directions of Matthews (1962) and of Crowe and Matthews (1964a, b) It was generally found more difficult to detect static fusimotor fibres influencing secondary endings than to find fibres affecting primary endings, because the acceleration in steady discharge of secondary endings by constant fusimotor stimulation was often quite small (see Table 1) About one third of all secondary endings could not be activated by stimulating ventral root filaments, but in many cases several (maximally four) fusimotor fibres influencing the same secondary ending were isolated they were always of the static type. For each primary ending several static fusimotor fibres could usually be found. Occasional observations were made on fibres which affected more than one primary or secondary ending I these cases the fibres exerted static effects on all of the endings involved The latter observation is confirmative to those of other authors and the similarity of the qualitative effects exerted by the same fusimotor fibre on different primary or secondary endings seems firmly established (Brown, Crowe and Matthews 1965 Bessou, Laporte and Pagès 1966)

Results

Position sensitivity

The measurement of position sensitivity during fusimotor activation has been described earlier (Lennerstrand and Thoden 1968b) During static fusimotor activation the hysteresis in the steady state curve was not significantly changed by the static fibre stimulation (cf Fig 1)

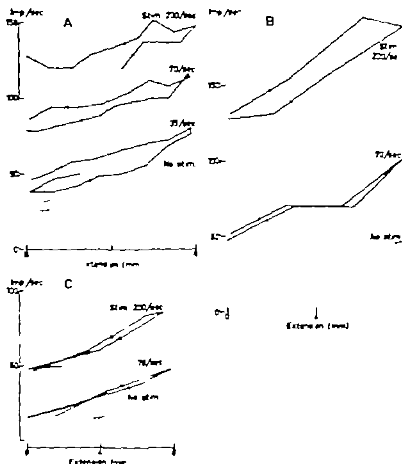


Fig 1 Steady state curves of endings deprived of fusimotor stimulation (dashed lines) and under static fusimotor activation (full lines) at the rates marked at the curves. A and B from two different primary endings. C from a secondary ending. The arrows show the direction in which the values are obtained, i.e. the sequence of increasing or decreasing lengths.

Two types of static fusimotor effect

In Fig 1 examples are given of the different actions on the position sensitivity obtained by stimulating static fusimotor fibres. With respect to their effects the static fibres seemed to constitute two groups. In some cases the position sensitivity of the ending was only insignificantly altered (less than 0.5 imp/sec mm) at any frequency of stimulation (Fig 1 A), whereas other fibres mediated an increase in the position sensitivity at increasing rates of stimulation (Fig. 1 B and C).

Both effects have been observed previously by Jansen and Matthews (1962b) on primary and secondary endings of decerebrate preparations with intact fusimotor innervation, by Whitteridge (1959) and by Harvey and Matthews (1961) on activation by single fusimotor fibres of unknown type, and by Crowe and Matthews (1964) on activation of primary endings by identified static fibres. Whitteridge (1959) claimed that in the extrinsic eye muscles of the goat the sole mode of action of fusimotor fibres was to increase the static position sensitivity of spindle endings.

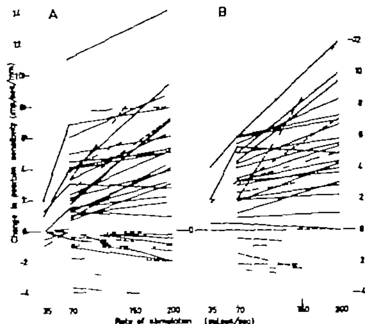


Fig. 2. Effects of static fusimotor activation on the position sensitivity. *A.* Primary endings. *B.* Secondary endings. Each line represents the action of a single static fibre on one spindle ending. The ordinate is the change in position sensitivity from the value in the non-activated state. On the abscissa are marked the stimulus frequencies applied to single static fibres. Solid lines are extensor endings, hatched lines flexor endings.

Fig. 2 is a compilation of all the static fusimotor actions on the position sensitivity observed in this study. The ordinate values of the diagrams are the changes in position sensitivity from the value of the non-activated ending induced by the stimulation of a single static fibre. On the abscissa the rates of stimulation are marked. Each line represents the action of one static fibre. Several lines coincide partly. Values of primary endings are plotted in Fig. 2 *A* and the values of secondary endings are given in Fig. 2 *B*.

As indicated by Fig. 2, the overlap was rather small between the two types of static fusimotor effects on the position sensitivity. The curves in Fig. 2 connecting positive values, nearly all had positive slope values. This means that almost all endings, in which the position sensitivity was increased at a low rate of static fibre stimulation, showed a further increase at higher stimulus frequencies. On the other hand, the curves connecting negative or small positive values of change in position sensitivity had slopes of negative or zero values: these endings showed no increase in their position sensitivity with increasing rate of static fibre stimulation. Moreover a *t*-test comparison between ordinate values well above zero to those around and below zero in Fig. 2 *A* at the rates of stimulation of 70 and 200 shocks/sec respectively showed that highly significant differences ($P<0.01$) existed between these two groups. It therefore seemed justified to subdivide the static effects into two

types no change or a decrease in the position sensitivity of an ending will be called the *type I static eff ct* whereas an increase in the position sensitivity will be termed the *type II static effect* In the case of one ending being influenced by several static fibres, both types of fusimotor effects were often observed, as will be reported in greater detail later on.

It must be emphasized that this grouping refers not to the static fibre as an anatomical entity but to the effect of stimulating the fibre on the response of an individual ending A static fibre might possibly have different effects on different endings even within the same spindle depending *e.g.* on the differences in the properties of the intrafusal muscle fibres or on the type of fusimotor nerve terminal. This idea could not be adequately tested in our experiments the material contains no well studied examples of one static fibre influencing more than one ending How ever close examinations of the records from experiments, relevant from this point of view published by Crowe and Matthews (1964*b* Fig. 3) and by Bessou, Laport and Pagès (1966 Fig 1) reveal both type I and type II effects on the different primary endings which responded to stimulation of one single static fibre On the other hand, the records available in the publication by Appelberg, Bessou and La porte (1966) all show that the same (type II) effects on stimulating a single static fibre affecting both a primary and a secondary ending of the same tenuissimus spindle (their Fig. 1 and 2) Thus for a single muscle spindle, it is not known whether or not one static fibre common to both the primary and the secondary endings of that spindle may exert different type of effects.

It should be observed that the estimations on position sensitivity from the record of the above mentioned papers had to be based on impulse frequency values measured 0.5 to 1 sec after completion of a stretch during constant static fibre stimulation and not on values obtained during stimulation with the spindle at constant length. Probably this did not invalidate the classification founded on the changes in position sensitivity since the magnitude of the decay in impulse discharge rate that occurs later than 0.5 sec after completion of stretch is thought to be very small (Crowe and Matthews 1964*a* the impulse frequency at 0.5 to 1 sec after stretch is therefore to the largest extent an expression of the position response at that particular spindle length.

Although it has not been definitely established that different actions on the position sensitivity can occur on different endings of the same spindle by stimulating one static fibre it seems only logical to refer to the effects themselves rather than to the fibres exerting them Expressions such as static fibre of type I effects, static fibre evoking type I effects static type I action, static activation of type I type I activation, type I action fibre *etc.* will therefore be used synonymously in the following

Acceleration of steady discharge by static fibre With respect to the changes in steady discharge or position response at zero extension no statistically significant difference could be noted between the two kinds of static fusimotor actions on endings of the same type This is shown in Table I for endings in extensor muscles

TABLE I Averages values and standard deviations of the increase in steady discharge zero extension by stimulating static fusimotor fibres classified as type I or type II according to their action on the position sensitivity of the endings. Spindle endings i extensor muscles are listed. Mea values and standard deviations in impulses/sec. Rat of static fibre stimulation in shocks/sec. I secondary endings too few type I effects were seen to merit tabling.

	Primaries				Secondaries	
Rate of stimulation	Stimulation type I		Stimulation type II		Stimulation type II	
	Mea ±S.D	n	Mean±S.D.	n	Mean±S.D.	n
35	37±15	6	40±29	4	31±24	6
70	65±24	13	63±28	21	41±32	19
200	98±37	13	118±60	21	77±53	16

Extensor secondaries were seldom influenced by fibres with type II effects. In flexor secondary endings, on the other hand, both actions could be observed (see below) The increase in position response caused by type I or type II activation of flexor secondaries were equal in magnitude. The position responses of primary endings were generally increased more than those of secondaries by static fibre stimulation t a defined rate in spindle endings of both extensor and flexor muscles (see also Appelberg, Bessou and Laporte 1966)

Observations on driving. Static fibres, but not dynamic fibres, has been observed to produce 'driving' of the discharge of the endings, i.e. each stimulus pulse evoked one pulse of afferent discharge (Crowe and Matthews 1964b Appelberg Bessou and Laporte 1966) In our f ΔL curves, 'driving' showed up as les l of constant discharge rate equal to the frequency of fusimotor stimulation. I occurred both in steady state curves (Fig 1 B) and in f-ΔL diagrams to triangular muscle length changes of low elocities (Fig. 3 B) t the rates of stimulation of 35 and 70 pulses/sec, but was never seen when stimulation at 200 pulses/sec was applied. Static fibres of type II effects were found to driv the afferent discharges more often than type I action fibres. I primary endings driving by static fibres evoking type II actions was observed in 9 out of 16 endings that could be studied in this respect, bu only in 2 out of 12 cases when primary endings were activ ed by static fusimotor fibre with type I effects. I secondary endings driving was less commonly seen on static stimulation producing type II effects it was elicited only in 2 cases out of 12. 'Driving' was never observed th ra her few occasions of type I activation of secondary ending

Distribution f type I and II effects t endings n d fferent muscles Table II presents the distribution of static type I and II effects to primary and secondary endings in extensor and flexor muscles. Both types of endings in flexor muscles were more commonly innervated by static fusimotor fibres w th type I ctio n than by fibres with type II effects, whil the reverse was tru for th ending in extensor muscles In fact tatic type I action was very rare n secondary ndings of extensor muscles.

For the extensor primaries the distribution shown in Table II is at variance with the one reported in the preliminary note (Lennerstrand and Thoden 1967) This is

TABLE II. The number of static fibres yielding type I or type II effects in spindle endings of extensor and flexor muscles. The total number of endings activated by static fusimotor fibres is also given in each column, as well as the approximate ratio of type I to II effects in each sample.

	Extensors		Flexors	
	Primaries	Secondaries	Primaries	Secondaries
Endings	20	11	8	7
Type I actions	15	1	9	9
Type II actions	21	19	4	4
Ratio I:II	3:4	1:19	2:1	2:1

because the preliminary results were based on a much smaller material of static fusimotor effects than was later available.

As mentioned earlier individual static fibres to the same ending could affect the position sensitivity differently. When more than three static fibres influenced the same primary ending of flexor or extensor muscles, usually both type I and II effects were seen. The only type I effect observed in an extensor secondary ending was recorded from an ending which also showed type II responses to other static fibres. Three other extensor secondaries had multiple static fusimotor innervation, all fibres with type II effects. In the flexor muscles one secondary ending was controlled by four static fibres which all had type I action and in two other multiply innervated secondaries static fusimotor performance of both types were seen.

In this context it is of interest to note that in the papers of Appelberg, Bessou and Laporte (1966) and Brown, Engberg and Matthews (1967) all the records of fusimotor activation of secondary endings in the tenuissimus and soleus muscles, respectively show type II effects.

Velocity sensitivity

From the relations between the velocity responses of an ending and the input velocity an expression of the velocity sensitivity can be given. In endings activated by static fusimotor fibre stimulation, the response related to the dynamic part of the 'triangular' length input applied in this study consists exclusively of the velocity response (Lennerstrand and Thoden 1968*a*). This conclusion was reached by showing that spindle endings lack specific sensitivity to acceleration and that all transients in the dynamic response during static fibre stimulation were really velocity dependent.

When activated by static fibres all endings fired impulses in response to length decrement (see also Crowe and Matthews 1964*b*, Jansen and Rudjord 1965). Velocity responses could thereby be calculated to both the stretch and the release phase of the movements. In case of primary endings this is in contrast to the conditions in the non-activated state or during dynamic fibre activation, when impulse firing is usually lacking during release of stretch.

Also in the velocity response of an ending under static fusimotor control, a 'quick' and a 'slow' part could be separated (cf Lennerstrand 1968) Functional differences between individual static fibres to primary endings were observed in the changes with velocity of the 'slow' velocity responses during stimulation. Thus, during activation both of the static fibres yielding type I action and of those giving rise to type II effects, generally an increase in the 'slow' velocity response with increasing velocity was seen at velocities above 1 mm/sec. This type of velocity response which was by far the most common, was observed in all the secondary endings. However in four extensor primary endings, activated by static fibres of type I action the 'slow' velocity response (to length increment) did not change with increasing input velocity in these exceptional endings stimulation of other static fibres resulted in slow velocity responses which increased with input velocity

Thus, the subdivision of the static effects that could be made from the velocity responses did not coincide with that performed from the effects of static fibre stimulation on the position sensitivity Since so very few units belonged to the latter type of slow velocity response, a labelling of the two different actions on the velocity response seems unnecessary The effects will be referred to as the 'common' and the 'exceptional' velocity responses during static fusimotor activation.

1 Common velocity responses during static activation

Typical f/ΔL diagrams are shown in Fig 3 of a primary ending activated by a static fibre of type II action, in Fig. 4 of a secondary ending influenced by a type I

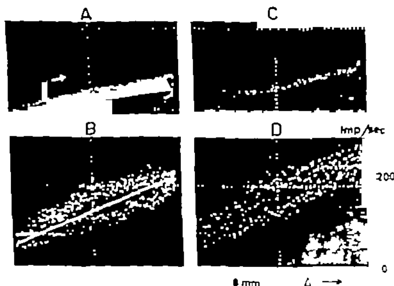


Fig 3 f/ΔL diagrams of an extensor primary ending to ramp-like changes of length of 2 mm/sec (A and B) and of 8 mm/sec (C and D) in the non-activated state (A and C) and during stimulation at 70 pulses/sec of a static fibre with type II action (B and D) ... lines are the approximate steady state curves. Arrows show ... movement in the time Length increases to the right in the pictures. Not... driving of ... fferent d...

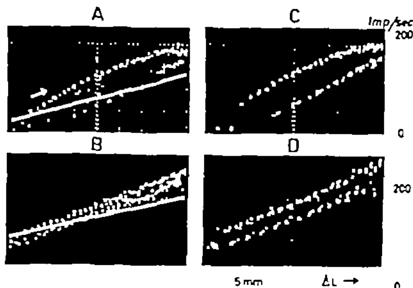


Fig. 4. f ΔL diagrams of flexor secondary ending to length changes of 5 mm/sec in A and B and to 5 mm/sec in C and D in the non-activated state (A and C) and during static fibre type I activation at 0 pulses/sec (B and D). Markings as in Fig. 3

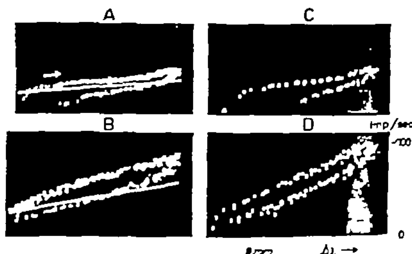


Fig. 5. f Δ diagrams of an extensor secondary ending to changes of length of mm/sec (A and B) and 8 mm/sec (C and D). The ending is deprived of fusimotor activation in A and C and stimulated by route of static fibre with type II action at the rate of pulses/sec in B and D. Markings as in Fig. 3

effect fibre and in Fig. 5 of a secondary ending activated by root of a static fibre with type II effects on the position sensitivity.

Qualitatively there was little difference between non-activated secondary endings and endings either primary or secondary under static activation: the ... response followed the variations in input velocity in a similar manner; the ... responses in static activation contained a quick part of the 'S' type, a 'quick P'

velocity response was seldom recognized (for definitions of quick P and S components see Lennerstrand 1967). The velocity responses to length increment and decrement became almost equal during static activation even in the primary endings. The quick and the 'slow' velocity responses were estimated in the same way as the corresponding components in non-activated secondary endings (Lennerstrand 1967).

From a comparison of the f/ΔL curves during static fusimotor activation in Fig. 3 B and D and the curves in Fig. 4 B and D and in Fig. 5 B and D some characteristic differences between primary and secondary endings emerge. The width of the f/ΔL loops of primary endings was usually larger than that of secondary endings, indicating a larger velocity sensitivity in the primary endings even during static activation. Primary endings showed a larger variability in their interspike intervals during static activation than in the non-activated state and more so than did the secondary endings (see also Matthews and Stein 1965). This is seen as a larger scatter of the points in the f/ΔL diagrams of the primary endings. In primary endings the interspike intervals usually varied more when the ending was activated by means of a type I fibre than when a type II fibre was stimulated.

Quick velocity response. The secondary ending of Fig. 5 has been chosen to illustrate typical static fusimotor effects on the quick component of the velocity response (Fig. 6 A). The thick line in the diagram of Fig. 6 represents the values obtained in the non-activated state. The quick response is seen to be mostly unchanged or reduced by the static fibre stimulation.

In the primary endings a direct comparison could not be achieved between the quick velocity responses in the non-activated state and during static fusimotor activation because of differences in measuring the quick component in the two cases (cf. Lennerstrand 1967). In turn, this rested on differences in the responses to

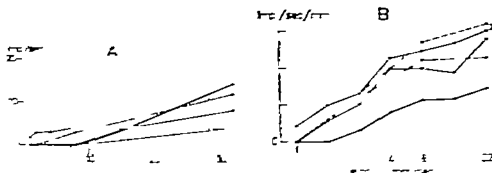


Fig. 6. A. Quick velocity responses of the secondary ending in Fig. [illegible] against velocity. Thick line labelled 0 represents values in absence of [illegible]. Lines above and [illegible] static activation [illegible]. B. [illegible] velocity responses of the same ending against velocity [illegible] in A.

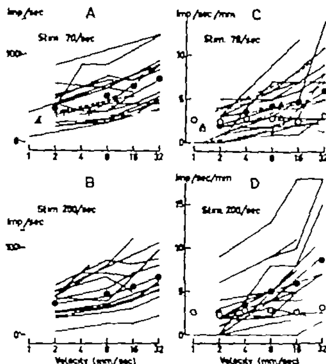


Fig. 7 Velocity responses of primary endings d ring static fibre stimulation plotted against log elocity Each line represents the action of one static fibre on one primary ending. Solid lines are extensor ndings, broken lines flexor endings. A and B, 'Quick' velocity responses. Rate of stimulation 70 pulses/sec in A and 200 pulses/sec in B. Filled circles are the me n values of e tensor endings t each locity filled triangles means of flexor endings. C and D 'Slow' velocity responses. Rate of stimulation 70 pulses/sec in C and 200 pulses/sec D Circles nd tria gles mark average alues of extensor and flexor endings respectivel filled symbols during static activation, open symbol in the non-activated st t

muscle shortening which in the non-activated state was characterized by a rapid cessation of impulse firing Therefore the absolute values of the velocity responses have been plotted in both primary and secondary endings instead of the changes in velocity response induced by the fusimotor activation as it was done when dealing with dynamic fusimotor effects (Lennerstrand and Thoden 1968b) Values of quick velocity responses of populations of primary and secondary endings in flexor and extensor muscles are in Figs 7 and 8 plotted against log values of velocity (in order to increase the scale at low speeds) Average values of extensor and flexor endings are represented by filled circles and triangles respectively The q k e locity responses were generally larger in primary endings (Fig 7 A and B than in secondary endings (Fig 8) but showed the same approximately linear relat n to log input velocity

In secondary endings the quick responses can be com pared before and d ring stimulation. The average alues of the secondary endings in absence of act vation are marked in Fig 8 by pen symbols. The quick responses were mostly f nd to be reduced by the static fibre timulation. Neither in primary nor in second end ings any systematic ariati ns in quick response were seen that corresponded t the type I and II grouping deri ed from the effects on position sensitivity

Slow velocity respons In the secondary ending of Fig. 5 the slow com ponent (Fig 6 B) is typically seen to increase with the rat f tim ilation t particular input velocity The linear increase against log velocity of the passi ending is maintained also in the activated tate

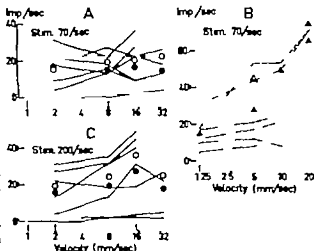


Fig. 8. 'Quick' velocity responses of secondary endings during static activation plotted against log velocity. A. Extensor endings at a rate of stimulation of 70 pulses/sec. B. Flexor endings at 70 pulses/sec. C. Extensor endings at 200 pulses/sec. Filled symbols are mean values during fusimotor stimulation, open symbols mean values in the non-activated state.

During static fusimotor activation the 'slow' velocity responses were very similar in primary and secondary endings both with regard to the magnitude and the variation with input velocity. In both types of ending the 'slow' component increased almost rectilinearly when plotted against the input velocity on a log scale, as can be seen for primary endings in Fig. 7 C and D and for secondary endings in Fig. 9.

The 'slow' velocity responses were often seen to be larger at a particular input velocity and rate of stimulation when a type II static fibre was activated than in the case of type I fibre activation, although in the present rather small number of experiments this difference, seen in both primary and secondary endings, was not statistically significant.

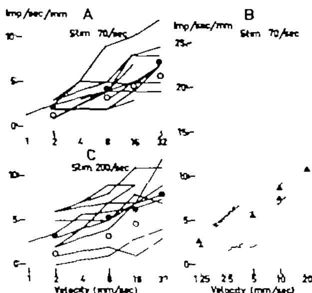


Fig. 9. 'Slow' velocity responses of secondary endings during static activation, plotted against velocity on a log scale. A. Extensor endings stimulated at 70 pulses/sec. B. Flexor endings at 70 pulses/sec. C. Extensor endings at 200 pulses/sec. Symbols as in Fig. 8.

The effect of static fusimotor activation to increase the 'slow' velocity response above the value of the passive ending, as shown in Fig. 7 *C* and *D* and in Fig. 9 was in both primary and secondary endings controlled by static fibres of both type I and type II. Average values during stimulation are represented by filled symbols and mean values in the non-activated state marked by open symbols. Primary endings, which in the non-activated state had constant 'slow' velocity response at input velocities above 0.5 mm/sec, showed the largest increase in 'slow' response but a change to higher values of the 'slow' component can clearly be seen also in the secondary endings.

Velocity responses of flexor endings. Of the flexor endings, which in Fig. 7, 8 and 9 are represented by broken lines, some had larger velocity responses than the rest of the endings of the same type in both extensor and flexor muscles. The endings with high velocity sensitivity were all located in the short peroneus muscle in which the spindles were exposed to relatively larger extensions than the spindles located in other muscles (*cf.* Lennerstrand 1968).

Variations in the initial extension and the amplitude of the length changes. The question whether any length dependence might occur during static activation in the response considered as velocity response was studied by shifting the operating point of the 'triangular' muscle movement or its amplitude. It was found that neither the quick nor the slow velocity responses were influenced by any of these procedures. This is illustrated by the typical results in Fig. 6 from an experiment in which the limit was shifted to 3 mm larger extension. Full lines represent control values and hatched lines the values obtained at an increased initial extension.

Exceptional' velocity responses during static activation

These velocity responses were obtained in four different primary endings situated in extensor muscles on stimulating one type I static fibre to each ending. The primary endings also showed the common velocity responses when activated byroots of other static fibres. The exceptional responses contained a 'quick P' component, at least to length increment. Usually the slow velocity responses to length decrement were smaller than those to length increment. 'Quick S' velocity responses were sometimes observed to length decrement, but because of differences in slopes of the *f*-ΔL diagrams to length increment and decrement, they could not be measured in the usual way (Lennerstrand 1968).

Typical *f*-ΔL diagrams of one of these endings are presented in Fig. 10. The quick P velocity response was measured only in response to length increment in the way described earlier (Lennerstrand 1968). In Fig. 11 *A* the quick P response has been plotted against input velocity. It is reduced below the value of the non-activated ending by the fusimotor stimulation at the highest rates, but somewhat increased at the lower velocities by stimulating the fibre at 35 pulses/sec. The rapid shift in impulse frequency at maximal extension in Fig. 10 *B*, *C* and *D*, interpreted as a quick P response to length decrement, is also reduced in magnitude with increasing rate of stimulation.

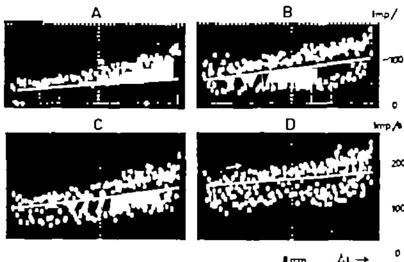


Fig. 10 f-ΔL diagrams of an extensor primary ending during static type I activation yielding the 'exceptional' type of velocity response. Speed of muscle length change 8 mm/sec in all pictures. *A* Non-activated ending. *B*. Static activation at 35 pulses/sec *C* at 70 pulses/sec and *D* at 200 pulses/sec. Markings as in Fig. 3

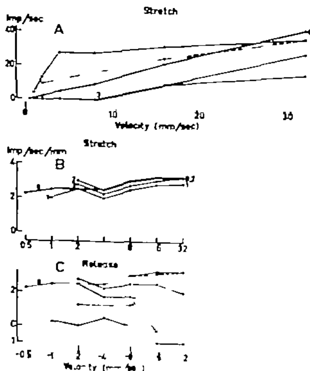


Fig. 11 Velocity responses of the ending in Fig. 10. *A*. 'Quick' velocity responses to muscle stretch plotted against velocity. Thick line labelled 0, represents values in the non-activated state. Lines marked 1, 2 and 3 represent values during static activation at 35 shocks/sec, 70 shocks/sec and 200 shocks/sec respectively. Full lines are control values, broken lines are from experiments in which the initial muscle extension was increased 2.5 mm. *B* 'Slow' velocity responses to stretch against log. velocity. Labelling and lines as in *A*. *C* 'Slow' velocity responses to release of stretch against log. velocity. Thin line, labelled 0 represents the responses of the non-activated ending to stretch. Other markings as in *A*.

The slow velocity response to length increment of the ending of Fig. 10 has been plotted against log input velocity in Fig. 11 *B*. With respect to the absolute values and the changes with input velocity the 'slow' velocity responses of this primary ending and, as a matter of fact of all endings with 'exceptional' velocity response were the same both in the absence and in the presence of static fusimotor activation.

Fig. 11 *C* shows the relation between the slow responses to length decrement and the input velocity. It is seen that with high rates of stimulation at a low input velocity the slow responses to length decrement reached the same values as was obtained to length increment. However during other experimental conditions the values to length decrement were considerably lower. Equal slow velocity responses to length increment and decrement could be attained at all input velocities if the initial muscle extension was increased at the same time as the ending was stimulated vigorously over the static fibre. This is indicated by the broken line labelled 3. The increase in initial muscle extension did not influence the 'quick' or the 'slow' velocity responses to length increment as can be seen from Fig. 11 *A* and *B* but reduced the quick P' responses to length decrement. The total velocity response taken as the area above or below the steady state curve circumscribd by the $f/\Delta L$ curves, became identical in length increment and decrement at large initial extensions and high rates of stimulation. Otherwise, the velocity response to length decrement was always larger than that to length increment (see Fig. 10).

Comparison of dynamic $f/\Delta L$ curves of primary and secondary endings during type I and II activation

It seems generally held that the impulse frequency responses of spindle endings are directly related to the intrafusal visco-elastic forces acting on the sensory terminals of the endings (*cf.* Matthews 1964). In order to widen the basis for the subsequent discussion on spindle mechanisms to generate the responses during static fusimotor activation, parameters of elasticity and viscosity of different endings as revealed by the $f/\Delta L$ diagrams have been compared in a scatter diagram (Fig. 12). The slope of the $f/\Delta L$ curve marked on the ordinate is taken as an expression of the elastic forces and the width of the diagram on the abscissa, as a correlate to the viscous forces acting on the ending. Extensor primary and secondary endings are plotted. The diagrams contain values from more endings than are shown in Fig. 7, 8 and 9. The input velocity has in all cases been 8 mm/sec and the rate of fusimotor stimulation 70 pulses/sec.

It is seen from Fig. 12 that the primary endings (marked by circles) and the secondary endings (triangles) differ very little with respect to the slope values, while the width of the $f/\Delta L$ diagram of a primary ending usually is much larger than that of a secondary ending although some overlap occurs. This would mean that the viscous forces acting on terminals of primary endings are generally larger than those acting on secondary terminals. No difference can be observed between the dynamically obtained $f/\Delta L$ diagrams of primary or secondary endings controlled by

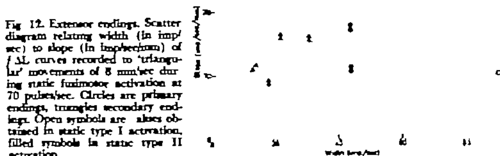


Fig 12. Extensor endings. Scatter diagram relating width (in imp/sec) to slope (in imp/sec/mm) of f ΔL curves recorded to 'triangular' movements of 8 mm/sec during static fusimotor activation at 70 pulses/sec. Circles are primary endings, triangles secondary endings. Open symbols are values obtained in static type I activation, filled symbols in static type II activation.

static fibres with type I (open symbols) or type II (filled symbols) effects on the position sensitivity

4 *Endings of intermediate type*

In the non-activated state the endings of intermediate type have characteristics of primary endings in their response to length increment and of secondary endings to length decrement (Lennerstrand 1968) In the present material they have been included among the primary endings. During static fusimotor stimulation, 8 type II effects and 3 type I effects were observed on the position sensitivity of extensor intermediate endings. Thus, the proportion of the two effects to intermediate endings is intermediate to that seen in extensor primaries and secondaries (Table II) The velocity responses of intermediate endings were all of the common type. Hence, the velocity responses to length increment and to length decrement became identical when these endings were activated by static fusimotor fibres.

Discussion

The most important new results of this investigation on muscle spindle responses during constant activation of single static fusimotor fibres are 1) the static fusimotor effects on the position sensitivity were found to form two distinct types. In type I the position sensitivity was unchanged or decreased, and in type II it was increased the distribution of the type I and II effects was different to the flexor and extensor muscles the flexor endings were much more commonly influenced by static fibres with type I effects than extensor endings the secondary endings in extensor muscles hardly ever received static fibres with type I effects 3 the f ΔL curves to 'triangular' changes of length of all the secondary endings and of the absolute majority of the primary endings had a close resemblance to those of non-activated secondary endings during static fusimotor activation the two kinds of endings showed only quantitative differences in their velocity response 4) If the velocity

response the quick component was reduced and the slow component was increased by the static fibre stimulation.

In the following discussion some motor control aspects of the results will be presented but the full discussion on these matters will be postponed until the intended analogue simulation of the spindle has been performed. An interpretation of the results, based on the present knowledge of spindle structure will also be attempted.

Some servotechnical implications

The static fusimotor fibre system seems to influence the function of muscle spindle endings as length and velocity measuring devices in several ways. It changed the operating point of the spindle ending by augmenting the position response. It varied the gain of the input-output relations not only by altering the position sensitivity of the endings but also by changing the dynamic gain operated by the slow velocity response. The quick velocity response is the genuine velocity response in the sense that it represents a derivative action in the stimulus-response relations. It was mostly reduced in magnitude. Some of these actions have been shown earlier with methods different from ours (Crowe and Matthews 1964*a*, Appelberg Bessou and Laporte 1966 Brown, Engberg and Matthews 1967)

The exceptional type of velocity responses during static fibre activation of primary endings, which appeared only in a few extensor spindles, seem by their paucity to be of very small importance for the control function of the spindles in the muscles investigated. They are perhaps more commonly occurring in other muscles, in which case their significance has to be reconsidered.

The possible functional importance of the variations in distribution of types I and static fusimotor effects to spindle endings in flexor and extensor muscles will be discussed at a later stage of this investigation.

It is probably of considerable significance that by means of static fusimotor activation, primary endings are enabled to signal position and velocity also during muscle shortening i.e. during muscle contraction in natural movements. This is in contrast to the behaviour of the non-activated and the dynamically activated primary ending since under these conditions their velocity sensitivity to shortening is so large that they usually cease to fire almost immediately and remain silent during the phase of muscle shortening (*cf* Lennerstrand and Thoden 1968*b* also for references) Information on position and velocity can, however be achieved also from the spindles of the antagonist muscles

Possible spindle mechanisms involved in static fusimotor action

The qualitative resemblance between the velocity responses of primary and secondary endings during static fibre stimulation would suggest common spindle mechanisms to generate the responses. Since the dynamically obtained responses are so similar to those of non-activated secondary endings and since it is known that secondary endings innervate nuclear chain fibres only (Barker and Cope 196_ Boyd 1962) it is

tempting to propose that the nuclear chain fibres are the site of origin for the spindle responses during static fusimotor activation. This is also the view favoured by Matthews and co-workers (Jansen and Matthews 1962*a*; see also Brown and Matthews 1966).

The differences in quick velocity responses between primary and secondary endings activated by static fibre stimulation, could arise from the disparities in the location of their sensory terminals on the nuclear chain fibres (Barker and Cope 1962; Boyd 1962). Primary endings have sensory terminals on structures centrally located in the fibre. These structures are supposed to contain less viscous elements than the poles of the fibres where the secondary endings terminate (*cf.* Matthews 1964). On these grounds the primary endings would be expected to have larger velocity responses than secondary endings (*cf.* B. H. C. Matthews 1933; P. B. C. Matthews 1964). That this is the case is indicated by the larger width of the $f/\Delta L$ curves of primary endings than secondary ones (see Fig. 12).

However, from recent anatomical studies on the efferent innervation of muscle spindles in the cat (*cf.* Barker 1967, also for references) there are reasons to believe that static fusimotor effects on primary endings can derive also from nuclear bag fibres which, in addition, are thought to convey dynamic fusimotor actions. Confirmative of these ideas are results from physiological studies on rabbit spindles, which contain only nuclear bag fibres (Barker and Hunt 1964). Nevertheless rabbit primary endings may show responses to fusimotor activation characteristic also of static fusimotor effects (Emonet Dénand, Laporte and Pagès 1966). The dynamic properties of the nuclear bag fibres must be altered quite markedly by fusimotor stimulation in order to mediate both fusimotor actions.

In the cat, features differentiating between the static fusimotor effects of nuclear bag and of nuclear chain fibres, respectively, should be searched for in the responses of primary endings, which innervate both nuclear bag and chain fibres. The static fusimotor effects on the primary endings of this study varied with respect to the changes induced both in the position sensitivity and the velocity sensitivity, but the variations in the two entities occurred independently of each other. Firstly an attempt will be made to use variations only in position sensitivity as a basis for the differentiation. By examining the records published by Emonet Dénand, Laporte and Pagès (1966) it was observed that all the static fusimotor fibres to the primary endings of the rabbit caused a decrease in the position sensitivity, *i.e.* they were of type I effects. It is also known that dynamic fusimotor fibres in the cat, which probably innervate nuclear bag fibres, reduce the position sensitivity of primary endings (Lennerstrand and Thoden 1968*b*). The type I static effects could therefore be proposed to signify static innervation of nuclear bag fibres. The type II effects would then indicate nuclear chain fibre participation. This idea would suit the results on the distribution of the two effects to primary and secondary endings if only endings in extensor muscles had to be considered. However, the occurrence of numerous type I effects found in flexor secondary endings does not seem to fit the above assumption. Flexor secondaries are believed to have their sensory terminal

organized in the same way as in extensor muscles, although this has not been verified for the particular flexor muscles used in this study.

Another proportion between nuclear bag and chain participation during static fusimotor activation could be proposed from the velocity responses. The velocity responses of primary endings during static fibre stimulation referred to as "exceptional" may possibly arise in nuclear bag fibres: they resembled the response of the non-activated primary endings in that the "slow" velocity response did not change with input velocity and in that a "quick P" response could usually be recognized. The idea that nuclear bag fibres are involved is further supported by the fact that the velocity response to length decrement was mostly different from that to length increment. However, this type of velocity response occurred too rarely to account for all the static fusimotor effects which might be expected to be mediated by nuclear bag fibres. In fact, the static fusimotor effects on primary endings in the rabbit are much more common (6 times) than the dynamic fusimotor effects. Thus at the present stage of our knowledge, it does not seem possible to ascribe with certainty the static fusimotor effects to nuclear bag or nuclear chain fibres respectively.

Some suggestions will also be forwarded on how the different static fusimotor effects on the position sensitivity might be generated, if a strict correlation to a particular type of intrafusal fibre cannot be made. One possible explanation would be that the type I and II effects depend on the location of the sensory terminals on the intrafusal fibres. It may be proposed that the terminals by being centrally or peripherally situated could perceive differently the changes in intrafusal tension set up in external changes of spindle length during the intrafusal fibre contraction. Another possibility would be that local responses in different sensory terminals of the ending located on different intrafusal fibres and concomitantly activated by the static fibre stimulation, would interact to give the afferent response of type I or type II. It is well known that the same efferent fibre can innervate several intrafusal fibres in the same spindle (Boyd 1962, Barker and Ip 1966). These two suggestions could eventually be tested in experiments on isolated spindles. Yet another possibility perhaps more easily tested would be that the static type I and II actions are correlated in some way to fusimotor fibres with "trail" and "plate" motor endings respectively (for a description of the motor endings see Barker 1967). In this case histological investigation to compare the efferent innervation in flexor and extensor muscles might tell whether there exists any relevant differences in the proportions between the different types of motor endings that could account for the physiological findings.

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References

Appelberg, B., P. Bessou and Y. Laporte. Action of static and dynamic fusimotor fibres on secondary endings of cat spindles. *J Physiol.* (Lond.) 1966, 185, 160–171.
Barker, D. The innervation of mammalian skeletal muscle. In *Myotatic, Kinesthetic and Vestibular Mechanisms.* Ciba Found. Symp. Editors A. V. S. de Reuck and J. Knight. Churchill, London 1967, pp. 3–15.

BARKER, D and M. COPE, The innervation of individual intrafusal muscle fibres. In *Symposium muscle re ptors*. Editor D BARKER. Hong Kong Univ Press, Hong Kong 1962, pp. 263—269

BARKER, D and J P HUNT Mammalian intrafusal muscle fibres. *Nature* (Lond.) 1964 *203* 1193

BARKER, D and M. C. I Sprouting and degeneration of mammalian motor axons in normal and de-afferented skeletal muscle. *Proc roy Soc B* 1966 *163* 538—554

BESSOU P Y LAPORTE and B PAGÈS, Similitude des effets (statiques ou dynamiques) exercés par des fibres fusimotrices uniques sur les terminaisons primaires de plusieurs fuseaux, chez le Chat. *J Physiol.* (Paris) 1966. *58* 31—39

BOYD, I A., The structure and innervation of the nuclear bag muscle fibre system and the nuclear chain muscle fibre system in mammalian muscle spindles. *Phil. T ans. B.* 1962. *245* 81—136.

BROWN, M. C., A. CROWE and P B C. MATTHEWS, Observations on the fusimotor fibres of the tibialis posterior muscle of the cat. *J Physiol.* (Lond.) 1965. *177* 140—159

BROWN, M. C., I ENGBERG and P. B. C. M TTHEWS, Fusimotor stimulation and the dynamic sensitivity of the secondary ending of the muscle spindle. *J Physiol.* (Lond.) 1967 *189* 545—550.

BROWN, M. C. and P B. C. MATTHEWS, On the sub-division of the efferent fibres to muscle spindles into static and dynamic fibres. In *Co trol and innervation of skeletal muscle* Editor B L ANDREW Thomson and Co Dundee 1966, pp. 18—31

CROWE, A. and P B. C. MATTHEWS, The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles. *J Physiol.* (Lond.) 1964*a*. *174* 109—131

CROWE, A. and P. B. C. MATTHEWS, Further studies of static and dynamic fusimotor fibres. *J Physiol.* (Lond.) 1964*b* *174* 132—151

EMONET DÉNAND F Y LAPORTE and B. PAGÈS, Fibres fusimotrices statiques et fibres fusimotrices dynamiques, chez le lapin. *Ar h. ital. Biol.* 1966 *104* 195—213

HARVEY R. J and P B. C. M TTHEWS, The response of de-efferented muscle spindle endings in the cat's soleus to slow extension of the muscle. *J Physiol.* (Lond.) 1961 *157* 370—392.

JANSEN, J K. S. and P B. C. MATTHEWS, The central control of the dynamic response of muscle spindle receptors. *J Physiol.* (Lond.) 1962*a*. *161* 357—378.

JANSEN, J K. S and P B. C. MATTHEWS, The effects of fusimotor activity on the static responsiveness of primary and secondary endings of muscle spindles in the decerebrate cat. *Act physiol. scand* 1962*b* *55* 376—386

JANSEN J K. S. and T RUDJORD, Fusimotor activity i na flexor muscle of the decerebrate cat. *Act physiol. scand* 1965 *63* 236—246.

LENNERSTRAND, G., Position and velocity sensitivity of muscle spindles in the cat. I Primary and secondary endings deprived of fusimotor activation. *Act physiol. scand* 1968. *73* 281—299

LENNERSTRAND, G and U T ODEN Fusimotor effects on position and velocity sensitivity of muscle spindles. *Experientia* (Basel) 1967 *23* 205—206.

LENNERSTRAND, G and U THODEN, Dynamic analysis of muscle spindle endings in the cat using length changes of different length-time relations *Act physiol. scand*. 1968a. *73* 234—250.

LENNERSTRAND G and U THODEN Position and velocity sensitivity of muscle spindles in the cat. II Dynamic fusimotor single-fibre activation of primary endings. *Acta physiol scand* 1968b. *74* 16—29

M TTHEWS, B H. C Nerve endings in mammalian muscle *J Physiol* (Lond.) 1933 *78* 1—53

MATTHEWS, P B C., The differentiation of two types of fusimotor fibre by their effects on the dynamic response of muscle spindle primary endings. *Quart J p Physiol* 1962 *47* 324—333

MATTHEWS, P B C., Muscle spindles and their motor control *Physiol Rev.* 1964 *44* 219—288.

MATTHEWS, P B C. and R B. STEIN, Differences in variability of discharge frequency between primary and secondary muscle spindle fferent endings of he cat *Natur* (Lond.) 1965. *208*. 1217—1218.

WHITTERIDGE, D The effect of stimulation of intrafusal muscle f bres sensitivity to stretch of extra ocular muscle spindles. *Quart J exp Physiol* 1959 *44* 385—393

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Exercise and Arterial Pressure during Simulated Increase of Gravity

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Abstract

Linnarsson D and G. Rosenhamer. *Exercise and arterial pressure during simulated increase of gravity* Acta physiol. scand. 1968 74 50—57

Heart rate and arterial pressure were continuously recorded in eight healthy young adults in the sitting position t 3 G acting i the head-seat direction, before and during leg exercise t 600 kpm/min for 6 min. I spite of the threefold increase of the hydrostatic pressure gradient along the head-trunk line the rterial mean pressure at the level of the heart was higher t 3 G tha at normal gravity both at rest nd during exercise Exercise t 3 G resulted in more rapid rise i the arterial mean and pulse pressures, the final increments being similar or somewhat greater than they were t normal gra ity The results emphasize the importance of the leg muscle pump as a booster of the cardiac pump in situations here large hydrostatic pressures in the lower portion of the body would otherwise tend to curtail e effecti circulating blood olume

In the upright body position the force of gravity causes an intravascular loss of blood" into the dependent portion of the low pressure system, resulting in impairment of the cardiac filling (*cf* Gauer and Thron 1965) Intermittent movements of the leg muscles counteract this effect through mechanical translocation of blood toward the heart However leg exercise also tends to increase the volume of blood contained locally within dilated muscular and cutaneous circ its (for review see Folkow Heymans and Neil 1965) A series of reports from this laboratory (Rosenhamer 1967 a, b, Bjurstedt, Rosenhamer and Wigertz 1967) have dealt with the effects of leg exercise at a sim lated increase of the force of gravity to three times its normal value This condition is characterized by the combined effects of a threefold increase of the effective weight of the blood and an exercise-induced widening of the muscle essels in the dependent parts of the body It was observed that the cardiac output for a given oxygen uptake was about 3 l/min lower than during exercise at normal gravity (Rosenhamer 1967 b) reflecting impaired ventricular filling Nevertheless, moderate leg exercise was capable of preventing circulatory collapse at 3 G which frequently became imminent after 5—12 min of

motionless resting at the same G level. This indicates a substantial increase of the arterial perfusion pressure at head level and thus of the systemic arterial pressure. The present investigation was undertaken to study the behaviour of the arterial pressure as influenced by constant-load leg exercise performed during exposure to 3 G

Material and methods

Eight healthy male young students were studied. Dimensional and functional data are given in Table I All were familiar with the subjective sensations experienced during centrifuge runs. Resting blood pressures and heart rates recorded during the experiments indicated no significant interference by anxiety or other emotional factors.

Experiments were performed in human centrifuge (radius = 7.4 m) where the effects of graded leg exercise in the sitting position could be investigated with the subjects exposed to force of 3 G nits acting in the head-to-seat direction (in the following this force and that of normal gravity are referred to as "3 G" or "high G" and "normal G" respectively) Details of the experimental conditions hav been described in previous reports (Bjurstedt and Rosenhamer 1967 Rosenhamer 1967 b)

Two experiments were performed on all subjects, one at normal G and the other t high G separated by 30 min rest. I each experiment measurements were made during 9 min rest, immediately followed by 6 min exercise at 600 kpm/min. I 4 subjects the normal G experiments preceded the high G experiments, the sequence being reversed for the rest of the subjects. They were all instructed to abstain from straining or other anti-G maneuvers. G-suits were not used.

The arterial pressure was recorded from the radial artery at the wrist, where Teflon catheter (O.D = 1.3 mm, I.D = 0.9 mm) was inserted percutaneously (Barr 1961) and connected to strain-gage manometer (Statham, P 23 A) mounted in the centrifuge cabin with its movable mass transversely to the G ector Because of the practical problems involved in recording accurately the arterial pressure in exercising subjects under simulated increase of gravity the technique used is described in some detail.

The pressure changes at heart level wer of primary interest. Therefore, an auxiliary manometer of the same type as the arterial manometer was connected to an ascendent tube of translucent plastic (I.D = 4 mm) the upper part of which was taped ertically onto the front of the sternum. Saline was then introduced in the auxiliary manometer system so that its meniscus was t the level of the insertion of the 4th rib t the sternum. This level was chosen to indicate the level of the heart. Clearly with the head of the auxiliary manometer positioned in the same plane as that of the arterial manometer the hydrostatic component produced by the intervening fluid column between the heart and the recording manometer

TABLE I. Individual data

Subj. No.	Age years	Height cm	Weight kg	PWC_{170} kpm/min
1	24	179	64	1150
2	22	183	70	1050
3	22	169	58	1050
4	23	183	65	1500
5	27	172	60	950
6	22	183	70	1100
7	24	173	65	800
8	19	180	66	1300

PWC_{170} = physical working capacity at pulse rate 170 (Sjöstrand 1947 Wahlund 194

was then always recorded by the auxiliary manometer irrespectiv of changes in G level or in body position. Hence to obtain the mean arterial pressure t heart level from the arterial manometer assuming linear characteristics and equal sensitivity for the two manometers, only the simple operation of subtracting the reading of the auxiliary manometer was required.

By means of T\ monitor it was possible to measure continuously during the experiments the level of the eyes in relation t the meniscus i.e the reference level for arterial pressure and thus lso t estimate the concurrent blood pressure t eye level (pressure t reference level minus the effectiv hydrostatic distance to ey level)

The signals from the two transducers were recorded continuously before and throughout each experiment on photokymographic paper at speed of 1.22 mm/sec via light beam galvanometers. The dynamic response of the arterial catheter-manometer-galvanometer system was uniform up to 9 cps, which was considered sufficient for obtaining reasonably accurate recordings of systolic and diastolic pressures. The arterial mean pressure obtained from the transducer output by an electronic integrating circuit was recorded simultaneously with the above pressures via separat galvanometer

I addition the heart rate was continuously recorded by an instantaneous cardiotachometer (Sturm and Wood 1947)

Results

A in previous studies under conditions similar to the present (Rosenhamer 1967 a, b, Bjurstedt, Rosenhamer and Wigertz 1967) the exposure to 3 G was well tolerated when combined with leg exercise. By contrast, the 9 min resting period at 3 G was experienced as relatively unpleasant and fatiguing and was accompanied by visual symptoms (see below) In accordance with earlier observations (Rosenhamer 1967) it was found that once exercise was commenced, any visual impairment subsided within 10 to 15 sec. In two subjects a progressi e fall in arterial pressure and rise in heart rate, combined with loss of peripheral and, finally central vision necessitated commencement of exercise before 9 min had elapsed in one (No 3) fter 5 min, and in the other (No. 5) after 6 min.

Arterial pressure at heart level

Fig. 1 typifies the continuous photokymographic recordings from which the present data were deduced. All arterial pressure recordings were first calibrated to refer to the level of the heart by subtracting the hydrostatic pressure recorded by the auxiliary manometer (see above "Techniques") Group means of individual time-averages over successive 15-sec intervals, and the standard error of these values, were then calculated and plotted against time

Fig 2 depicts the time courses of the arterial systolic, diastolic and mean pressures during the latter part of the resting periods and throughout the exercise periods, at normal G (left side of graph) and at 3 G (right side) It can be seen that the arterial mean pressure during rest at 3 G was higher than at normal gravity During the first 5 min of the resting period at high G (not shown in the graph) the average mean pressure attained a stable level about 18 mm Hg over the resting level at normal gravity A gradual fall occurred however during the last 4 min of the resting period

A marked increase in the arterial mean pressure followed the onset of exercise both at normal and high G In all cases at the 3 G level there was a rapid initial pressure rise within the first 3—4 sec, followed by a transient but marked fall

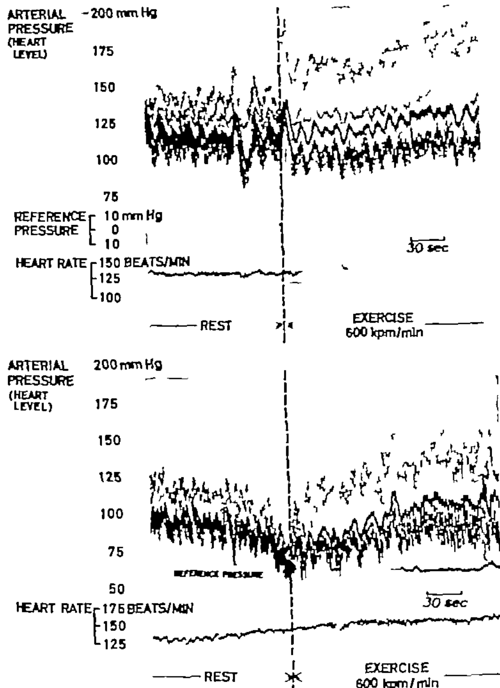


FIG. 1 Segments of original photokymographic records (scale = 1/7 of original) obtained from two different subjects showing heart rate and systolic and diastolic, as well as mean arterial pressures as recorded from the right radial artery during rest and exercise at 3 G. Reference pressure indicates magnitude of hydrostatic pressure changes due displacement of reference (heart) level during the course of the experiments. T obtain true" arterial pressures heart level the reference pressure (scale inserted in upper panel) should be subtracted.

Upper panel. subject with slight visual impairment ("grey-out") at the end of the period. Arterial mean and pulse pressures remain relatively stable throughout this

Lower panel. subject reporting complete loss of vision, which was preceded by fall in mean and pulse pressures.

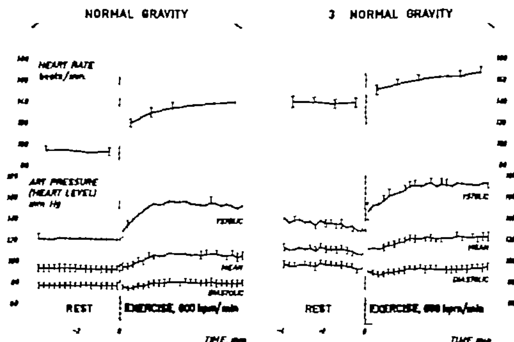


Fig 2. Time courses of hanges in heart rate and arterial pressure at heart level (recorded from the right radial artery) during rest and leg exercise (600 kpm/min) t normal gravity and at 3 G The ertical lines represent 1 SE about the means. I the resting condition the arterial mean pressure t heart lev l was 15—18 mm Hg higher t 3 G than t normal gravity with a gradual fall toward the end of the resting period. Exercise resulted in more rapid increase in the mean and pulse pressures t 3 G than t normal gravity the final increments being simila or somewhat greater t 3 G

(Fig 1 upper panel) Throughout the exercise period the mean pressure remained higher at 3 G than at normal gravity ($P<0.001$) During the 4th min of exercise at high G the a erage mean pressure attained a relati ely stable final alue of 122 mm Hg which was approximately 15 mm Hg higher than that obtained at normal gravity The range for individual time-averages over the last two minutes of exercise at high G was 107—138 mm Hg

The pulse pressure showed a gradual decrease during rest at high G especially toward the end of the resting period (Fig 2) Following the onset of exercise it rose much more rapidl at high G than it did at normal gravity It attained a maximum by the end of the 2nd min and then remained essentially constant ntil the end of the exercise period During the last 4 min of exercise th pulse pres re was slightly greater t 3 G than at normal gra ity At both G levels the pulse pressure changes during exer ise wer predominantly due to changes in the systolic pressure (Fig 2)

After the completion of exercise t 3 G b th the mean pres ure and pulse pres re fell relatively slowly to atta n th ir pr -exercise alues only after several minutes.

Heart rate

Fig 2 shows the heart rate changes plotted as group means of individual time averages over successive one-min periods. The heart rate just prior to commencement of exercise averaged 92 beats/min at normal G and 144 beats/min at high G It rose by 23 beats/min (to 167) from rest to the 6th min of exercise at high G as compared to 48 beats/min (to 140) at normal G From the 3rd to the 6th min of exercise there was an increase by 7 beats/min (range 4 to 12) at high G and by 5 beats/min (range 0 to 6) at normal G

Blood pressure and vision

The gradual decrease in the arterial mean pressure and pulse pressure during the latter part of the resting period at high G (Fig 2, *cf* Fig 1 lower panel) was accompanied by progressive visual impairment in 6 of the 8 subjects. In the two subjects (Nos. 3 and 5) who reported complete or nearly complete loss of vision, the systolic and mean arterial pressures, when referred to the level of the eyes (see "Techniques") fell below 25 and 10 mm Hg, respectively before the exercise was initiated. After 15 sec of exercise the group means for systolic and mean pressures at eye level had increased by approximately 20 and 5 mm Hg respectively Within this period of time any visual symptoms had already subsided, and there was no re occurrence of visual symptoms during the remaining exercise period. (Whereas all recorded pressures have so far been referred either to heart or to eye level, it should be noted that the terms "systolic" and diastolic pressure as used in this report reflect the dynamic situation only at the site of measurement in the radial artery)

Discussion

Before discussing the results of the present study it should be noted that the exposure to increased G caused small artefacts in the calculated arterial pressure at heart level due to slight caudad displacement of the plastic tube connected to the auxiliary manometer (see above "Techniques) The displacement of the meniscus in the tube was observed and measured against a projection scale on the screen of the TV monitor and was less than 5 mm in the G axis on changing the G level from 1 to 3 G Another type of error is likely to have occurred under increased G through downward displacement of the heart relative to the framework of the thoracic cage. Roentgenographic studies have shown that the greater force of 5 G increases the distance between the base of the skull and the heart by less than 10 mm (Rushmer 1947) and since the two types of errors tend to cancel each other the present technique may be assumed to give a accurate picture of 1) the mean arterial pressure and its changes at heart level, and 2) the pulse pressure and its changes at the region of the radial artery

The exposure to 3 G in the present study must have caused an increase of the hydrostatic pressure gradient along the heart-sinus distance Under these conditions, the observed rise in the arterial mean pressure at heart level during both rest and

exercise over the values recorded at normal gravity may be attributed mainly to a reduced stimulation of the carotid sinus baroreceptors due to a hydrostatically induced pressure fall at the level of these receptors. The stimulation of baroreceptors located near the heart (mainly those in the aortic arc) on the other hand, should at the same time be increased, which would tend to depress the arterial pressure. The net effect on the mean arterial blood pressure viz. an increase at heart level emphasizes the stronger influence of the baroreflexes originating in the sinus regions. The possible influence of baroreflexes originating in other sites of the arterial system (Heymans and Neil 1958) and of mechanoreceptors in the low pressure system (see Gauer and Thron 1965) in the arterial pressure response to exercise under the present conditions remains open to question. A simple calculation shows that, with a shift from 1 to 3 G the pressure regulating mechanisms caused the arterial mean pressure to remain constant at some level about half way between the heart and the carotid sinus, whether the subject was exercising or not.

That the response of the heart rate to exercise was much more sluggish at 3 G than at normal gravity is in agreement with previous observations, and reflects the fact that most of the increase in cardiac output is brought about by adjustments in the stroke volume (Rosenhamer 1967).

The initial response of the arterial systolic and mean pressures at the transition from rest to exercise at 3 G (Fig. 1 upper panel) was in certain aspects similar to the typical blood pressure response at the onset of exercise in the sitting position at normal gravity as described by Holmgren (1956). However, whereas the duration of the initial increase and subsequent fall in the arterial mean pressure was approximately the same at normal gravity and high G the magnitude of the rise was much greater at high G (*cf.* Fig. 1). Both at normal gravity and at 3 G the instantaneous pressure rise may result from an involuntary short lasting Valsalva maneuver at the onset of exercise. Part of the rise may, however, also reflect an instantaneous increment in venous return accompanying the first strokes of the leg muscle pump.

The observation that the arterial mean pressure fell markedly after its initial rise is of special interest in view of the considerable reduction of the pulmonary blood volume that accompanies the exposure to 3 G at rest (Rosenhamer 1967 b). This pattern of response seems to support the assumption that the initial blood volume in the lungs is a limiting factor in the rapid increase of cardiac output as exercise is commenced and the flow resistance in the working muscles is instantaneously reduced (*cf.* Holmgren 1956).

As can be seen in Fig. the further rise in the systolic pressure and pulse amplitude was more rapid at 3 G than at normal gravity. This observation seems to favour the interpretation that a large volume of blood was redistributed into the intrathoracic spaces at the high G level through the action of the leg muscle pump, thus resulting in a more marked improvement of the ventricular filling and thus rise of the stroke volume. This is in agreement with previous observations that the response of the stroke volume to exercise becomes more marked when the magnitude of the G-force vector in the head-seat direction is increased (Rosenhamer 1967 b).

The rapid increase of systolic and mean pressures following the transition from rest to exercise emphasizes the importance of the leg muscle pump as a booster to the cardiac pump especially in situations where large hydrostatic pressures in the lower portion of the body would otherwise tend to curtail the required increase in effective circulating blood volume.

Whereas the arterial systolic and mean pressures decreased somewhat toward the end of exercise at normal gravity (*cf* Holmgren 1956) such decreases did not occur at 3 G From this one can conclude that large hydrostatic pressures in dependent parts of the body did not interfere with the homeostatic control mechanisms which maintained the systolic and mean arterial pressures at increased levels during exercise. That the pulse pressure remained essentially constant agrees well with previous observations that no systematic changes in the stroke volume occur between the 5th and 9th min of exercise at 3 G (Rosenhamer 1967)

References

Barr, P.-O Percutaneous puncture of the radial artery with multi-purpose Teflon catheter for indwelling use. *Acta physiol. scand.* 1961 *51* 343—347

Berne, R. M. and M. N Levy Heart. *A Rev Physiol* 1964 *26* 153—181

Bjurstedt H, G Rosenhamer and O Wigertz, High-G environment and responses to graded exercise. *J ppl. Physiol.* 1967 I press.

Craig F N and E. G Cummings Slowing of the heart t the beginning of exercise *J ppl. Physiol.* 1963. *18* 353—356

Folkow B C. Heymans and E Neil, Integrated aspects of cardiovascular regulation. In *Handbook f Physiology Circulation.* Washington, D C. Am. Physiol. Soc., 1965 sect. 2, *III* 1787—1823.

Gauer, O H. and H. L. Thron Postural changes in the circulation. In *Handbook of Physiology Circulation* Washington, D C. Am. Physiol. Soc 1965 sect. 2. *III* 2409—2439

Heymans C. and E. Neil, *Reflexogenic A eas f th Cardiovascular System* Churchill. London. 1958. pp. 271

Holmgren A., Circulatory changes during muscular work in man. With special reference to arterial and central enous pressures in the systemic circulation. *S and J clin. Lab Invest* 1956. *8* Suppl. 24 pp 97

Rosenhamer, G Antigravity effects of leg exercise. *Acta physiol. sc nd* 1968 *72* 72 -80

Rosenhamer, G Influence of increased gravitational stress on the adaptation of cardiovascular and pulmonary function to exercise *Act physiol scand* 1967 b Suppl 276. pp. 61

Rushmer, R. F A roentgenographic study of the effect of pneumatic anti-blackout suit on the hydrostatic olumns in man exposed to positive radial acceleration *Amer J Physiol* 1947 *151* 459—468.

Sjöstrand T Changes in the respiratory organs of workmen at an ore smelting works. *Act med. scand* 1947 *128* 687—699

Wahlund H., Determination of the physical working capacity *A med sc d* 1948 Suppl 215 pp. 78.

Acta physiol. scand. 1968. 74. 58—68

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Structures Essential for the Effect of Cholecystokinin on the Guinea Pig Small Intestine in vitro

By

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Abstract

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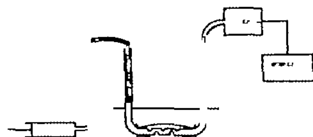
The stimulating effect of cholecystokinin on the small intestine was investigated by different registration techniques in the guinea pig ileum in vitro. The effect of cholecystokinin was blocked by atropine morphine and tetrodotoxin but not by hexamethonium or pretreatment with reserpine Cholecystokinin was still effective after desensitization to 5-hydroxytryptamine After a propagated contraction in the circular muscle layer induced by an increased intraluminal volume or by 5-hydroxytryptamine there was a refractory period for 20—60 sec which could not be registered when the corresponding response was induced by cholecystokinin. It is concluded that the stimulating effect of cholecystokinin on the two muscle layers of the guinea pig small intestine is mediated via a cholinergic neural pathway without cholinergic synapses. The mechanism of action is still unknown but does not seem to consist in a release of 5-hydroxytryptamine

A stimulating effect of cholecystokinin preparations on the motility of the small intestine was described by Jung and Greengard already in 1933 However not until a highly purified preparation of cholecystokinin was produced by Jorpes *et al.* in 1964 was it possible to demonstrate that this intestinal effect was due to the hormone itself and not to impurities in the preparations (Dahlgren 1966) In earlier experiments we have registered a stimulating effect of cholecystokinin on the longitudinal muscle layer of the small intestine in vitro (guinea pig) and on the peristaltic activity *in vivo (cat) (Hedner et al 1967) In both cases the intestinal effect could be* blocked by atropine, thereby differing from the effect on the gall-bladder Thus the effect on the small intestine might be suspected to be an indirect one. The aim of this investigation was to elucidate the structures necessary for the development of the stimulating effect of cholecystokinin on the guinea pig small intestine in vitro.

Material and methods

Guinea pigs of either sex, weighing [illegible]–300 g were used. They were killed by decapitation and a piece of the small intestine was immediately dissected out and immersed in Krebs solution in a bath thermostated at 3[illegible] °C The medium was bubbled with a mixture of 95 % oxygen and 5 % carbon dioxide The following types of preparations and recording systems were used

Fig. 1 Schematic drawing of preparation D. The gut is threaded on to U-bent, fenestrated polyethylene tube, the one shank of which is connected via thin catheter to syringe containing Krebs solution. The other shank is connected to pressure receptor Bath volume 100 ml.



A. The piece of small intestine was mounted vertically in a 25 ml bath in the common way The contractions of the longitudinal muscle layer were registered isometrically by a Wheatstone bridge transducer (Svema SG4—45) connected to potentiometer recorder

B The same preparation as A, supplemented with an intraluminal copper electrode and one electrode in the bath fluid, giving modification of the coaxial stimulation described by Paton (1954) simplified inasmuch as only the contractions of the longitudinal muscle layer were registered. Both the intestine and the intraluminal electrode were fixed to the bottom of the bath (volume 50 ml) The electrode stimulation consisted of square pulses, duration 1.6 msec, frequency 10 imp/sec. The voltage giving maximal contraction was titrated for each single preparation and the voltage used in the experiment that followed was chosen to 1.5—2 times the titrated value, in most cases 8 V. The contractions of the longitudinal muscle layer were registered isometrically by a force displacement transducer (FT 03) connected to a Grass polygraph recorder (Model 5 D)

C. The same preparation and recording as A, but the lower end of the gut was tied on to a glass tubing connected to container with a changeable fluid level. The upper end of the gut was tied tightly The intraluminal pressure could be changed and read, and a "peristaltic reflex" could be elicited. However only the contractions of the longitudinal muscle layer were registered in this simplified Trendelenburg preparation.

D For the registration of the contractile activity of the circular muscle layer the preparation shown in Fig. 1 was used. The gut was threaded over fenestrated polyethylene tube and ligated at the ends. One end of the tube was connected to volumetric low pressure transducer (Model PT 5 A) in turn connected to Grass recorder (Model 5 D)

Injection of fluid into the intraluminal compartment led to propagated contraction waves beginning at the oral end, forcing the fluid up in the shanks, where the movement of the fluid was registered as changes in the pressure. It was found that the intraluminal volume could be adjusted to give degree of distension that caused no spontaneous activity The upper level of the bath fluid was kept constant by continuous suction. When the bath was drained, the fluid level in the shanks decreased, the intraluminal volume increased, and propagated contraction was elicited. Thus both the size and the velocity of the increase of the intraluminal volume was simply and reliably standardized which seemed to be of importance for the reproducibility. A characteristic picture was registered with an initial drop in pressure followed by an increase eventually repeated (Fig. 3, 4 8) If the reflex was blocked, e.g. by hexamethonium, only the drop in pressure was seen which normalized on refilling the bath (volume 100 ml

Doses given for acetylcholine (Ach) and for histamine refer to the chlorides, for 5-hydroxytryptamine (5HT) to the creatinine sulphate. A preparation of cholecystokinin containing 250 Ivy dog units per mg was used for preliminary experiments The results were repeated with preparation containing 1500 Ivy dog units per mg The latter preparation is referred to in the following by the short form CCK.

Lyophilized tetrodotoxin, 100 μg per ampoule was obtained from Sank Co Tokyo Japan

Results

I Effect of pretreatment with reserpine A group of 10 guinea pigs was treated intraperitoneally with reserpine in doses increasing to 1 mg per animal and day after two weeks Two animals per week were sacrificed, and the effect of CCK on the

The preparations of cholecystokinin were supplied by Prof E. Jorpes Stockholm.

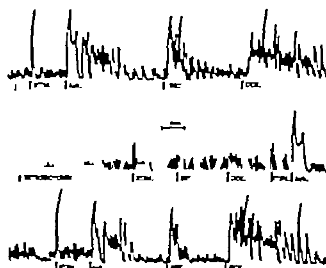


Fig. 2. Effect of tetrodotoxin on the responses to Ach, coaxial stimulation with 8 V (STIM) 5HT and CCK. The lower record shows the recovered responses after washing out the tetrodotoxin for 8 min. Preparation B, bath volume 50 ml.

small intestine was investigated, using preparation A. The response to CCK did not differ from that of control animals, not even after 5 weeks of treatment with reserpine.

The effect of reserpine was also investigated histologically with special reference to the enterochromaffine cells by the technique of Falck and Owman (1965).[2] A certain reduction of the frequency of these cells was seen, but still after treatment with reserpine for 5 weeks a considerable amount of enterochromaffine cells remained. After mechanical removal of the mucosa the responses to Ach and to CCK were reduced to the same extent compared with controls (preparation A). The following histological examination often revealed a small rest of mucosa in the preparation.

? *Eff t of t trod toxn* The results obtained in earlier investigations indicated a possible action of CCK by a nervous pathway. To check this theory we needed a denervated preparation. The simplest wa to achieve this in our types of preparation seemed to be the "pharmacological" denervation described to result from treatment with the fish poison tetrodotoxin (Gershon 1966). With preparation B, we found that tetrodotoxin in a concentration of 0.04 µg/ml in the bath completely abolished the responses to 5HT and CCK in submaximal doses, and the response to coaxial stimulation (Fig. 2). The responses to Ach and to histamine remained unaffected. After washing out the tetrodotoxin, all responses recovered within 10 minutes. Our experience of the effect of tetrodotoxin on the response to coaxial stimulation, histamine and Ach are in accordance with those reported by Gershon (1966) who however found only a partial reduction of the response to 5HT. With preparation D the effect of tetrodotoxin (0.05 µg/ml) corresponded t that found w th prepara-

[2] Kindly prepared by Dr. B. Falck, Lund.

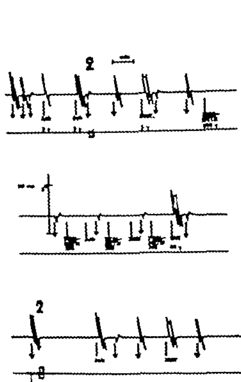


Fig. 3

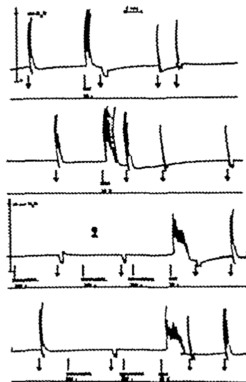


Fig. 4

Fig. 3. Effect of hexamethonium on the responses to increased intraluminal volume, Ach, histamine (HIST) and 5HT. The lower record shows the responses after washing out the hexamethonium for 3 min. The increase of intraluminal volume was made by draining the bath fluid, indicated by the arrows. Preparation D bath volume 100 ml.

Fig. 4. Effect of hexamethonium on the responses to increased intraluminal volume 5HT and CCK. The increased intraluminal volume was achieved by draining the bath fluid, indicated by the arrows. Preparation D bath volume 100 ml.

tion B with the exception that the propagated contraction otherwise elicited by a low dose of Ach or histamine (0.06 μg/ml) was also inhibited.

3 Effect of hexamethonium. As described earlier (Hedner *et al.* 1967) hexamethonium in doses up to 4 μg/ml did not affect the response of the longitudinal muscle coat to CCK. This observation was confirmed with preparation B. Nor was there any inhibition of the response to 5HT Ach or coaxial stimulation, when hexamethonium was present in the bath in the same concentration (4 μg/ml).

When the circular muscle layer was studied with preparation D hexamethonium in a dose of 2—4 μg/ml was effective to paralyse the propagated contraction elicited after increasing the intraluminal volume (Fig. 3). It is also apparent from Fig. 3 that Ach and histamine in low doses (0.05 μg/ml) elicited a response similar to that seen after increasing the intraluminal volume like 5HT (0.5—0.8 μg/ml) and CCK (0.03—0.1 U/ml) (Fig. 4). The responses to Ach and histamine were inhibited by



Fig 5. Effect of morphine on the responses t Ach, 5HT CCK and coaxial stimulation. About 2 mi fter washing out the morphine the response to coaxial stimulation had reappeared. Preparation B, bath olume 50 ml.

hexamethonium in the dose mentioned above (2—4 µg/ml) the responses to 5HT and CCK were not affected.

4 Eff ct of antiseroto in agent In the experiments mentioned above the effect of CCK seemed to be of the same character as that of 5HT Some antiserotonin agents were investigated as an attempt to elucidate whether the effect of CCK was mediated via 5HT or not

Lysergic acid derivatives, stated as the most potent inhibitors of 5HT were investigated in the form of methyl lysergic acid butanolamide It could, however hardly reduce the responses of preparation A to submaximal doses of 5HT and CCK when gi en in doses up to 5 µg/ml Higher doses of the antagonist caused a slowly increasing contraction of th preparation and did not permit any conclusions.

The dose of phenoxybenzamine (Gaddum and Picarelli 1957) necessary to inhibit the response to 5HT abolished that of CCK but also that of Ach

With morphine 0 0? µg/ml the responses of preparation B to submaximal doses of 5HT and CCK were completely blocked without any reduction of the response to Ach (Fig 5) However th response to coaxial stimulation was also inhibited by this dose of morphine

Some antihistamines ha e been reported to possess also an antiserotonin activity which does not always parallel their antihistamine potency (Doepfner and Cerletti 1957) If the effect of CCK were due to a release of 5HT both agonists might be expected to be inhibited to about the same degree by these antihistamine and antiserotonin agents. Antazoline promethazine and diphenylpyraline were selected to

represent different degrees of antiserotonin potency and were investigated with preparation B in this respect. Ach, histamine 5HT and CCK were given in doses adjusted to give equal submaximal responses. Electrode stimulation was performed as described above. The antagonists were left in contact with the preparation for 15 min before the effect of an agonist was tested. The doses of antagonists required to reduce the response to histamine were lower and showed smaller variations than when 5HT and CCK were concerned. As a rule, 0.05 µg/ml of prometazine or 0.06 µg/ml of diphenylpyraline completely inhibited the responses to 5HT and CCK. CCK seemed to be slightly more resistant to the antagonists. However these doses of prometazine and diphenylpyraline also completely abolished the response to coaxial stimulation and reduced that to Ach. Antazoline inhibited the response to histamine in a dose of 0.05 µg/ml but even after increasing the dose to 1 µg/ml there was practically no reduction of the responses to 5HT CCK, coaxial stimulation or Ach.

Atropine inhibited the response to Ach coaxial stimulation, 5HT and CCK completely in the dose range used for these agonists. The actual dose of atropine (0.01 µg/ml) did not affect the response to histamine

5 Desensitization to 5HT The attempts to separate the effects of 5HT and CCK with different inhibitors failed to solve the problem. Therefore we utilized the property of the preparations to develop a tachyphylaxis, primarily to 5HT With preparation A, 5HT was given repeatedly the substance being washed out before every new application, which was performed within 2 min. A gradual decrease of the response to the repeated administration of 0.4 µg 5HT per ml was seen, until there was no response at all. In this state, the preparation still retained its response to Ach and to CCK almost unchanged (Fig. 6) It proved possible to provoke a similar tachyphylaxis to CCK so that the response to the originally effective dose was reduced almost to zero. Now the response to 5HT was reduced to about 40 % of the original one The response to Ach remained unchanged.

The tachyphylaxis to 5HT developed faster if the substance was not washed out before every new application, meaning a cumulation of 5HT in the bath. When the response to 5HT in such an experiment had disappeared completely the responses to coaxial stimulation and to CCK were still unchanged (Fig. 7)

Instead of increasing the concentration stepwise of 5HT a large dose (5 µg/ml) was administered to preparation D and left in contact for 30 minutes. Then, 0.7 µg of 5HT per ml, previously effective in inducing a propagated contraction, was found to be inactive but the usual dose of CCK, 0.05 U/ml, still elicited the response. The responses following changing of the bath fluid Ach and histamine wer nhibited. After washing out the excessive dose of 5HT all th stimulants wer again effective

6 Relation of CCK to reflexly ind d at action P eparation C was used for investigation of contractions of the longitudinal muscle coat and preparation D for the circular muscle coat. In both preparations rhythmic contractions ould be licited by increasing the intraluminal volume. The border alue for the increase of volume required for a reflex proved to be quite constant nd eproducible in both prepara-

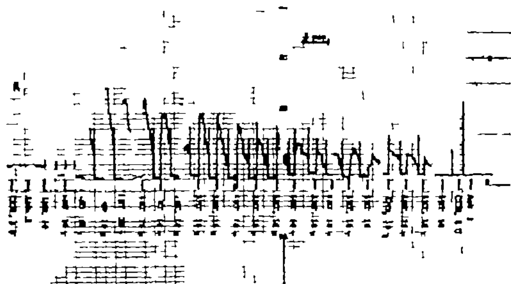


Fig. 6. Response to CCK and Ach after desensitization to 5HT. The bath fluid was changed after each drug application. Preparation A, bath volume 25 ml.

tions. With preparation C CCK in subliminal doses did not alter the increase in volume necessary for the reflex. Higher doses caused contractions similar to those obtained with preparation A, i.e. an increase in tone with smaller rhythmic contractions superimposed while the reflexly elicited response implied a rhythmic change between contractions and almost complete relaxation.

Nor preparation D became more sensitive to volume increase after subliminal doses of CCK. However when an effective dose of CCK was given a propagated

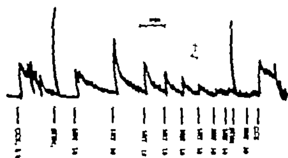


Fig. 7. Effect of CCK and coaxial stimulation after desensitization to 5HT. The bath fluid was not changed after the administrations of 5HT meaning a cumulation of this substance in the bath. Preparation B, bath volume 50 ml.

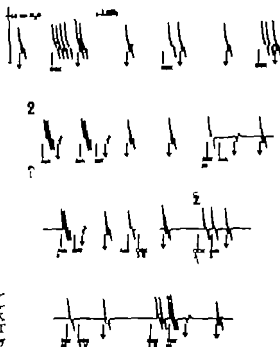


Fig. 8. Temporary failure of preparation D to respond to increased intraluminal volume, Ach, 5HT and CCK after a response elicited by Ach or 5HT but not after response licited by CCK. Bath volume 100 ml.

contraction was obtained, giving a similar rhythmic change of the intraluminal pressure as seen when a response was induced by increasing the intraluminal volume. Such a response was obtained also by 5HT and of low doses (0.05 µg/ml) of Ach or histamine which by experience were expected to give a contraction of the longitudinal muscle layer The responses elicited by increased volume, Ach and histamine were inhibited by hexamethonium, responses elicited by 5HT and CCK were not.

When a response was ind ced in preparation D g by increasing the intraluminal volume (simplest by draining the bath fluid as described above) there was a refractory period for up to 60 sec during which new response could not be elicited, neither by changing the bath fluid, nor by administration of Ach, histamine, 5HT or CCK. This refractory period was also observed after eliciting a response with Ach, histamine or 5HT However after a response elicited by CCK, no refractory period could be registered from 10 sec after the response had faded. The period before 10 sec could not be investigated with security (Fig 8)

Discussion

Our preparation of CCK contained 1500 units per mg and does not represent completely pure cholecystokinin. Still the results obtained were regarded representative for the hormone as previous investigations (Hedner et al 1967) showed that the intestinal effect of preparations with different degrees of purity (up to 4 000

units per mg) followed the unitage of cholecystokinin and not the amount of substance given.

The observation that atropine in low doses inhibited the response of the longitudinal muscle layer of the guinea pig small intestine to CCK (Naito *et al.* 1963, Hedner *et al.* 1967) was reproduced in this investigation and also found to be true for the effect on the propagated contraction of the circular muscle layer. Thus it seemed possible that CCK acted either via a direct release of Ach, perhaps over a nervous pathway, or by reducing a possible inhibitory catecholaminergic activity which might be suspected as Furchgott (1960) found both alfa- and beta-receptors in the small intestine. The considerable dosage of reserpine given for 5 weeks to our animals should be expected to inactivate such a catecholaminergic activity. As CCK was still active in preparations from these animals it is less probable that its mechanism of action consists of abolishing an inhibitory activity on the muscle cells of the gut mediated by catecholamines.

As CCK was effective when histamine was blocked (*e.g.* with antazoline) and histamine was effective when CCK was blocked (*e.g.* with atropine) an action over histamine receptors may be excluded. Instead, a direct release of Ach seemed to be the most probable mechanism. This theory gains support by the finding that the effect of CCK was inhibited not only by atropine but also by morphine, reported to inhibit the release of Ach from the nerve endings (Schaumann 1957). However it seemed unlikely that CCK could release Ach by a direct action at the nerve endings, as the effect was inhibited by the fish poison tetrodotoxin, reported to interfere with the impulse transmission in the nerve (Ishihara 1918). Thus an intact nerve transmission seemed to be necessary for the effect of CCK. There was no evidence that CCK sensitized the gut for afferent impulses from strech receptors activated by distension of the lumen, nor for a directly stimulating effect on such receptors.

The nervous pathway mediating the effect of CCK did not seem to contain any cholinergic synapses as the effect of CCK was not influenced by hexamethonium. Thus it seems reasonable to place the effect of CCK on a terminal cholinergic neurone in the nervous plexus of the small intestine or on a non-terminal neurone connected to the terminal one by a non-cholinergic synapse. Several authors have concluded the same localization for certain effects of 5HT consistent with the effects mediated via the so-called M receptors of Gaddum and Picarelli (1957). The other type of receptor for 5HT, the D-receptor, they concluded to be situated on the smooth muscle cell. This type of receptor seems to be of small significance in the small intestine (Gaddum and Hameed 1954). Also, phenoxybenzamine and LSD-derivatives reported to block the D-receptors (Gaddum and Picarelli 1957) produced non-specific blocking or otherwise inconclusive results in our preparations which is in accordance with results reported by Brownlee and Johnson (1963). The effect of 5HT mediated via the D-receptors seem to be negligible in our preparations as the doses of 5HT used by us were blocked by atropine, morphine and tetrodotoxin, indicating an effect via a nervous pathway.

In this situation it might be suspected that the polypeptide CCK acted by releasing

the smaller molecule 5HT analogous to other polypeptide hormones, e.g. certain pituitary hormones. To investigate this possibility the effects of antiserotonin agents were of interest. As pointed out above LSD-derivatives and phenoxybenzamine seem to be specific inhibitors only for the D-receptors which does not seem to be of significance in the preparation and dose range used by us. For the M receptors, no specific inhibitors have been described to our knowledge. Morphine and the antihistamines investigated abolished not only the response to 5HT but also that of coaxial stimulation. Their 5HT-blocking effects therefore seem to be non-specific. Thus the effects of CCK and 5HT had to be separated by some other technique. The tachyphylaxis that easily develops to 5HT in the small intestine in vitro is a well known phenomenon. It proved possible to extend it to a complete loss of responsiveness to 5HT without any noteworthy decrease in the response to coaxial stimulation. The ceasing effect of 5HT thus did not depend on an exhaustion of the ability to conduct nerve impulses or to release Ach at the nerve endings but seems to affect some structure in the neurone that is sensitive to 5HT. Also a single large dose of 5HT led to a similar block in preparation D. This is in accordance with the view of Brownlee and Johnson (1963) who stated that the only specific antagonist to 5HT found by them was 5HT itself.

As CCK was still effective after inducing a desensitization to 5HT in this way it may be concluded that responsiveness to 5HT is not necessary for the effect of CCK. Also the preserved response to CCK after treatment with reserpine may indicate that access to 5HT is not essential for the effect of CCK but does not mean any proof of this as only a reduction but not a complete depletion of 5HT was guaranteed. Another separation of the effects of 5HT and CCK was found with preparation D, where a refractory period was found after responses elicited by an increased intraluminal volume or by administration of Ach or 5HT but could not be registered after CCK. If CCK acted by a release of 5HT with an additional effect consisting of a protection against the refractory period, CCK might be expected to break the refractory period after a response to Ach or 5HT. However CCK like the other stimulants failed to be effective in this situation. Thus also this experiment supports the view that the effect of CCK is different from that of 5HT and probably not mediated via a release of 5HT.

The propagated contraction of the circular muscle coat obtained by Ach and histamine in preparation D was inhibited by hexamethonium. It seems reasonable to assume that this effect was reflexly elicited by contraction of the longitudinal muscle coat, the reflex pathway containing at least one cholinergic synapse like the reflex induced by increasing the intraluminal volume (Kosterlitz 1967). This response thus can be trigged off by a contraction of the longitudinal muscle coat as well as it can be trigged when this muscle layer is rendered inactive (Kosterlitz *et al.* 1956).

In conclusion, the stimulating effect of CCK on the two muscle layers of the guinea pig small intestine was found to be mediated via cholinergic neural pathway without cholinergic synapses. The mechanism of action is still unknown but does not seem to consist in a release of 5HT.

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References

BROWNLEE, G. and S. E. J..so. The ..t of the 5-hydroxytryptamine receptor on the intramural nervous plexus of the guinea pig isolated ileum. *Brit. J. Pharmacol.* 19.3 *21* 30.—322.

DAHL..., S. Cholecystokinin. Pharmacology and clinical use. *Acta ... scand.* 19.. Suppl. 3 7 2..—2.0.

DOEPFNER, W. and A. CERLETTI. Über den Serotonin-Antagonismus einiger Antihistaminika unter Berücksichtigung ihrer chemischen Struktur. *Arch. Int. Allerg.* 1957 *10* 348—354.

FALCK, B. and CH. OWMAN. A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Act. Univ. Lund.* 1965. II 7 1—23.

F..ACWORTH, R. S. Receptors for sympathomimetic amines. In *Ciba Found. Symp. Adrenergic Mechanisms.* Ed. J. R. Vane. Churchill 1960 246—252.

GADDUM, J. H. and K. A. HAMEED. Drugs which antagonize 5-hydroxytryptamine. *Brit. J. Pharmacol.* 19.4 *9* 24.—248.

GADDUM, J. H. and Z. P. PICARELLI. Two kinds of tryptamine receptors. *Brit. J. Pharmacol.* 195. *12* 323—328.

GERSHON, M. D. Effects of tetrodotoxin on innervated smooth muscle preparations. *J. Physiol. Lond.* 19.. *18.* 4P—5P.

HEDNER, P., H. PERSSON and G. RORSMAN. Effect of cholecystokinin on small intestine. *Acta physiol. scand.* 19.. *70* 250—254.

I..MANN, F. Über die physiologischen Wirkungen des Fucutouins. *Mitteil. med. F. ... Tokio* 1918. .. 3.5 .42.

JORPES, E. V. MUTT and K. TOCZKO. Further purification of cholecystokinin and pancreozymin. *Acta chem. scand.* 19.4 *18* 240.—.410.

IVY, F. T. and H. GREE... Response of the isolated gall bladder to cholecystokinin. *Amer. J. Physiol.* 1933 *103* 27.—278.

KOSTERLITZ, H. W., W. PIRIE and J. A. ROBINSON. The mechanism of the peristaltic reflex in the isolated guinea pig ileum. *J. Physiol. Lond.* 1956 *133* 681—691.

KOSTERLITZ, H. W. Intrinsic intestinal reflexes. *Amer. J. Dig. Dis.* 1967 *12* 245—254.

NAT.., S. R. I.. and T. SAT.. Études sur la cholécystokinine, son mode d'action sur la contraction de la vésicule biliaire. *Presse méd.* 19.3 *35* 2688—2689.

PATON, W. D. M. The response of the guinea pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol. Lond.* 1954 *127* 40P—41P.

SCH..., W. Inhibition by morphine of the release of acetylcholine from the intestine of the guinea pig. *Brit. J. Pharmacol.* 1.5. *12* 115—118.

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Afferent and Efferent Activity in the Renal Nerves of Cats

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Abstract

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In anesthetized cats efferent as well as afferent activity has been recorded in the kidney nerves. Pulse synchronous bursts of efferent impulses could regularly be recorded which after vagotomy and baroreceptor denervation were augmented and changed int continuous type of discharge. The efferent activity could be modified by electrical stimulation of peripheral sensory nerves. It was inhibited by elevation of systemic blood pressure by i. noradrenaline injection, and greatly increased by lowering of blood pressure by i. acetylcholine. I the same nerves afferent activity also occurred, in few instances spontaneously but more often after elevation of renal vein pressure, elevation of arterial perfusion pressure (by pump) mechanical pressure applied to the hilus area or local dilution of the blood by close intra-arterial injection of Ringer solution. Local ischemia for one minut or less elicited intense afferent activity. The results indicate that enous distension and increased subcapsular pressure are adequate stimuli for these afferent responses and that more than one kind of fibers are engaged in the afferent response. The functional significance of the afferent activity remains to be determined.

It is well known that the kidneys are abundantly supplied with nerves from the thoracolumbar sympathetic. The functional significance of this innervation is still uncertain. On the basis of histological observations the possibility has been discussed that some nerve fibers may influence tubular function but most authors seem to agree with Homer Smith (1956) in his concept that the only clearly demonstrated f nction of the renal nerves is that of vasoconstriction. Although it is generally accepted that during physiological crisis" the activity in the kidney nerves may markedly reduce renal blood flow and even cause nuria some differences of opinion seem to prevail regarding the role of the kidney nerves nder normal conditions Pappenheimer (1960) has concluded that under normal resting conditions n unanesthetized animals or man the sympathetic supply to th kidney is pparent l inactive although it may be activated by exercise postural ha ges et

The possibility that the kidney nerves may also contain afferent fibers has attracted but little attention although Pines (1960) reported that he kidney nerves of cats afferent impulses could be recorded which increased in intensity inverse propor

tion to the tone of the renal vessels and in direct proportion to the level of the systemic blood pressure.

In a recent study in this laboratory Åström and Crafoord (1967) found that afferent impulses could be recorded from the kidney nerves of rats when e.g. the renal vein pressure was elevated but not under ordinary resting conditions. It was concluded that adequate stimuli for the mechanoreceptors responsible for this activity were increased kidney tenseness (intrarenal pressure) and perhaps also distension of the intrarenal veins.

In the study to be reported here efferent as well as afferent activity in the kidney nerves has been recorded in cats under various experimental conditions.

Methods

The experimen wer carried out on total of 46 cats anesthetized with pentobarbital sodium (30—35 mg/kg or w th chloralose-urethane (60 plu 50—100 mg/kg) after ether induction. A tracheal cannula was inserted and jugular ein cannulated for intravenous injections. The left kidney w exposed by mid-line incision. In most experiments the renal ein was exposed by blu dissection to allow the application of an adjustable screw clamp by which the enous pressure could be gradually increased. In the experiment in which the kidney was perfused to this was accomplished by ttaching the pump (Sigmamotor Inc.) t the loop of polyvin l Tygon) and polyethylene tubing connecting the left renal rtery to the righ t femoral rtery. The sy temic arterial pressure was recorded from the left femoral artery nd the renal ei pressur from a catheter inserted int the spermatic (ovarian) ein. A Grass Model 5 Polygraph with strain-gauge transducers was used for these recordings. Renal blood flow was measured some instances by an electromagnetic flow probe (Statham Microflo® lectromagnetic flowmeter).

The renal nerves were dissected from the point of division of the renal artery in the proximal direction le lev l of the arterial branch t the suprarenal gland, at which point ganglion rule was observed. A binocula dissecting microscope was used. After placing the ner trand on the bipolar silver wire lectrodes, paraffin was used to prevent drying of the filament. A Grass P 6 preamplifier Tektroni 502 A oscilloscope and a Grass model C-4 amera w re used for the recording of the action potentials nd Grass Model S 4 stimula or for lectrical timulation of peripheral nerves.

Results

Eff t act ty wa regularl present in the proximal part of the kidney nerve cut just proximall t the point of division of the renal artery. The activity was synchronous with the h art beat (Fig. 1 A). Fig. 1 also shows the occurrence of slow wa es. These were not regularly observed, neither before nor during electrical stimulation, but are particularl marked in Fig. 1 A and B. These waves did not seem to be due to muscle art f t but probably depended on the setting of the amplifier which in these ases resulted in a slow return of the base line after an intense synchronized burst of impulses. Electrical timulation (20 p.p.s., d ration 3 ms 5 V for 3—5 sec) of peripher l sensory nerves (g the perficial radial nerve) in most cases caused an initial gmentation of the efferent activ ty (Fig. 1 B). When the stimulation was stopped there was regularly a complete inhibition of the efferent sympathetic activity f r ouple of sec onds (Fig. 1 C). Pinching a foot w th a pair of forceps produced a und ilent period" after release of the stimulus (Fig. 1 D). This response to somatic fferent ner tim lation persisted after vagot my (Fig.

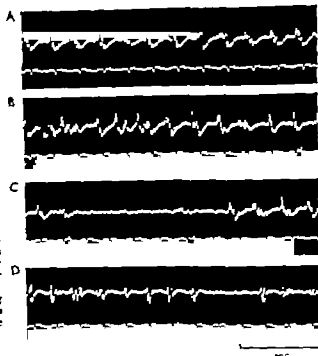


Fig. 1 Cat, chloralose-urethane anesthesia. Pulse synchronous ef ferent activity in the kidney nerv (upper curv) and ECG recording (lower curve)
A. Control.
B At signal (horizontal line) electrical stimulation (20 p.p s.) of the central end of the superficial radial nerv increased the activity particularly at the beginning of the stimulation period.
C When the electrical stimulation of the afferent nerve was stopped the efferent activity was completely inhibited for couple of seconds.
D This "silent period" following brief period of afferent nerve stimulation could also be elicited by pinching foot.

? A and B) The spontaneous activity greatly increased following blockade of the carotid sinus nerves (0.5 ml 0.25 per cent lidocaine solution bilaterally) in the agotomized animal and the activity changed into a more continuous type of discharge (Fig. C) In such preparations electrical stimulation of a peripheral sensory nerve still caused first an increase and, after release of the stimulation, a complete inhibition of the efferent activity The inhibition became evident about 300 msec after release of th stimulus both before and after vagotomy as well as after carotid baroreceptor denervation (Fig 2 D)

The efferent activity in the kidney nerve was also recorded by a different technique using the R-peak of the ECG to trigger the sweep of the oscilloscope From these experiments t was evident that the intermittent bursts of efferent outflow were strictly pulse synchronous (Fig. 3 A) By this technique the influence of intravenously injected noradrenaline and acetylcholine was studied. A few seconds after injection of 3 µg noradrenaline in animals with intact agus nerves and carotid baroreceptors there was a complete cessation of the efferent bursts of impulses (Fig 3 B) The activity returned to control level after bout two minutes (Fig. 3 C) Injection of 3 µg acetylcholine i.v greatly augmented the efferent outflow within a few seconds (Fig. 3 D) Five seconds after another injection f noradrenaline (3 µg) during augmented activity produced by acetylcholine the efferent activity was completely abolished (Fig 3 E) The changes in activity were well correlated in time with the increase and decrease in systemic blood pressure.

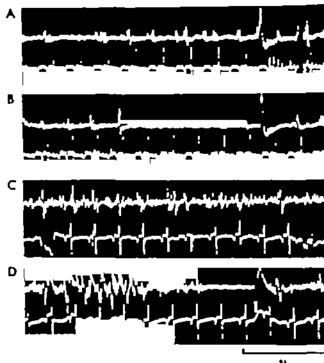


Fig. .. Same experiment as in Fig 1 (cont.)
A and B The response to lectrical timulation of the afferent nerve was essentially the same after vagotomy
C. After blockade also of the carotid baroreceptors the efferent activity was enhanced and changed int more continuous type of discharge
D Electrical stimulation of the afferent nerve still produced essentially the same response ... initial augmentation followed by silent period when the stimulation w stopped.

Affe e t ctivity was recorded in the same nerve as was used for the study of the efferent activity Thus, as a rule a portion of the nerve following one of the branches f the renal artery was placed on the electrodes.

Spontaneous afferent activity was present only in a few of the about 30 animals studied in this respect The impulse frequency when spontaneous activity was present was about 1 3 per second and the individual spikes had a duration of about 0.5—1.0 msec The afferent impulses occurring pontaneously always increased with elevation of renou pressure to about 10—15 mm Hg (Fig 4 B) If no spontaneous activity was present higher enous pressures of 15—40 mm Hg elicited an afferent response in several ases These results were obtained in kidneys with normal blood supply as well a in kidneys perfused *in situ* with blood from a femoral artery It was not possible t determine the conduction elocity of the fibers concerned.

Afferent activit could in several cases be elicited by mechanical pressure applied with a glass rod t the urf ce of thekidney close to the hilus (Fig 4 D) Pressure applied in a imilar manner to other part of the kidney surface was ineffective Stasis of or local pres re in the ureter did as a rule not produce any definite responses but sometimes response of different type was obtained (Fig 4 E) Elevation of the presu in th pelvis by Ringer injection is a catheter inserted into the ureter was als ineffectiv in most instances whereas in a few cases a response similar to that f llowing pinching or applying pressure on the reter w th a pair of forceps was observed as in Fig 4 F)

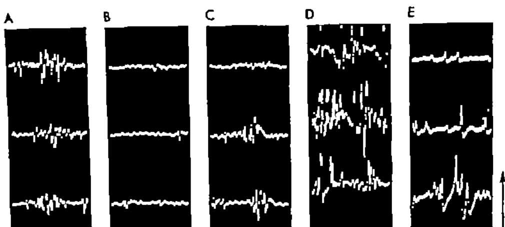


Fig. 3 Cat, chloralose-urethane anesthesia. Efferent activity recorded in the kidney nerv Individual sweeps triggered by the R peak of the ECG Recordings in this nd following figures to be read from below upwards.
A Control showing that the efferent bursts of impulses are strictly pulse synchronous.
B I. injection of noradrenaline (3 μg) abolished the efferent activity
C When systemic blood pressure falls the activity returns.
D When the blood pressure was lowered by I. injection of acetylcholine (3 μg) the activity was greatly enhanced.
E E en this enhanced activity could be completely abolished by another injection of nor adrenaline (3 μg)

In perfusion experiments the afferent activity was recorded under various conditions of flows and pressures. If the perfusion was stopped the local ischemia after 10 up to 60 sec ga e rise to massive afferent discharge which subsided when, after switching on the pump, the kidney had resumed ts normal color (Fig 4 F—H) which usually occurred within 3—5 sec. Increasing the perfusion pressure often augmented (or elicited) an afferent activity Fig. 5 illustrates an experiment in which a wide range of pressures was studied. At low flows (1—? ml/g and min) there was a teady activity of bout 5 spikes per second. When the perfusion pressure was elevated above 200 mm Hg the activity increased markedly and at a pressure of bout 250 mm Hg it was three times as high as at about 150—200 mm Hg When the pressure was reduced the impulse frequency remained high for some minutes and then gradually decreased.

In a further analysis of f ctors which might influence the afferent acti ty n the kidney nerves, Ringer sol tion was injected locally in the arter f the blood perfused kidney It has previously been shown that this procedure ill for some minutes increase the subcapsular pressure in the kidney A few sec nds after the lose intraarterial injection the perfusion pressure drops precip tousl due to the fall in peripheral resistance following the dil tion of the blood If afferent activity was present at the start of such an experiment (Fig 6 A) t usually disappeared as the perfusion pressure dropped (Fig 6 B) When the pressure then —ad

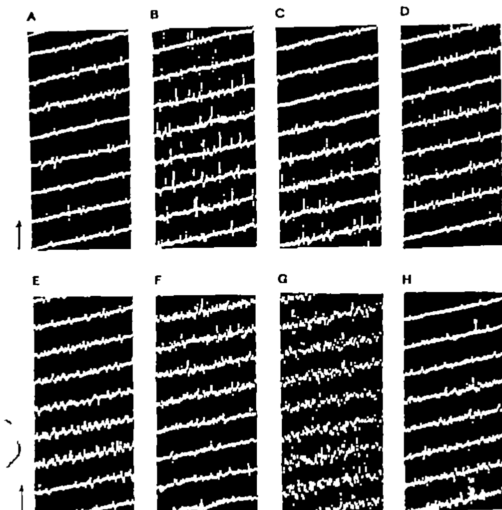


Fig 4 Ca pentobarbital anesthesia. Kidney perfused I situ t constant rate Each sweep corresponds to ?( ) msec Afferent activ ty be renal nerv (A) was augmented by enous pressure lev tion to 10 15 mm Hg pplied in B and released in C. Mechanical pressure applied to the hilus area w th glass rod elicited brief burst of impulses (D) Local pressure on the reter produced hange in base line activity (E) but no large spikes of the type seen after enous stasis or mechanic l pressure on the hilus area Stopping the perfusion pump first decreased F and then w hi 10—15 seconds caused massiv afferent activity (G) which arai disappe red hen the perfusion was re-started and the kidney resumed normal color (H

climbed the afferent activity ret rned and before the perfusion pressure had reached the pre-injection level the afferent activ ty was considerably enhanced (Fig 6C) This increased acti ity graually diminished during the following minutes while the perfusion pressure ret rned to pre-injection level which in this case was 110 mm Hg. The flow rat of the pump was kept constant (35 ml/min) throughout the test.

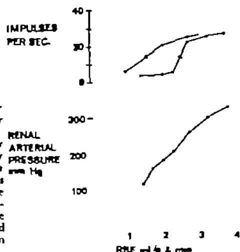


Fig. 5. Cat, pentobarbital anesthesia. Kidney perfused in situ. Afferent impulse frequency recorded from the renal nerv at different per fusion pressures obtained by adjusting the flow rate of the pump. When the renal blood flow (RBF) was increased it took some time before nerve activity was augmented. When flow was decreased enhanced activity remained for some time although the perfusion pressure fell approximately along the same curv as when the flow was increased. The pump was adjusted each minute and the pressure readings taken at the end of each one-minute period.

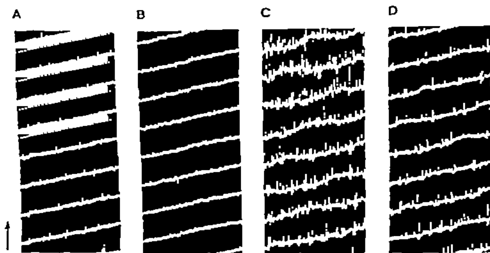


Fig. 6. Cat, pentobarbital anesthesia. Kidney perfused in situ. Each sweep corresponds t 700 msec. Close intraarterial injection of 1 ml Ringer solution (B) caused an immediate cessation of spontaneous activity present before the injection (A). A few seconds later and while the blood pressure was returning to control level, there was an intense ugmentation of the afferent activity (C) which gradually decreased. D recorded 20 sec after C.

Discussion

Many investigators (e g Pappenheimer 1960) ha e emphasized that under normal conditions the nerve supply to the kidney seems to be inactiv and apparently plays no important physiological role On the other hand, Schaefer (1960) has pointed out that the kidney is one of the best innervated organs, even more so than the heart. Sell t al. (1958) has demonstrated that lowering of the blood pressure of hy

nerventilation caused the same changes in the sympathetic outflow to the kidney as in other sympathetic nerves.

In recent years the efferent innervation of the kidney has attracted renewed interest in the discussion of the possibility of nervous control of renin release (Wander 1965 Castenfors 1967)

Action potentials synchronous with heart beats were first described by Adrian, Bronk and Phillips (1932) in different sympathetic nerves. Gernandt, Liljestrand and Zotterman (1946) found that the efferent impulses in the splanchnic nerves of cat were increased by hypoxia and elevated carbon dioxide content in the inspired air. Increasing the systemic blood pressure by adrenaline injection decreased the impulses in the splanchnic nerve whereas injection of acetylcholine increased the efferent discharge

The results obtained in this investigation have shown that the pattern of efferent activity in the renal nerves seems to be identical with that previously recorded in the splanchnic nerve. Our results and previous observations regarding sympathetic nervous outflow seem to support the following concept. *The basic discharge from the* vasomotor center which is enhanced by factors such as increased arterial P_{CO_2} and decreased P_{O_2} is of a continuous type (Fig. 2 C). This continuous discharge is intermittently inhibited by baroreceptor afferents and this modifies the basic outflow from the vasomotor center into a pulse synchronous pattern (Fig. 1). Decreasing baroreceptor inflow by lowering blood pressure greatly augments the sympathetic outflow (Fig. 3 D) whereas an increase of this inflow caused by blood pressure elevation may abolish the sympathetic activity altogether (Fig. 3 B)

The sympathetic outflow is also modified by sensory inflow from the periphery. In this investigation electrical stimulation of cutaneous sensory nerves as a rule produced a noticeable increase in sympathetic discharge which was always followed by a period of inhibition (Fig. 1). Apparently peripheral sensory inflow may both augment and decrease sympathetic outflow depending upon the type of afferent fibers activated as well as the stimulus strength (Fedina *et al.* 1966 Coote and Downman 1966. This conclusion is supported also by observations of differential vascular reactions following electrical stimulation of various afferent fibers made by several researches Sjöstrand 1960 Johansson 1962)

The observations that the basic pattern of sympathetic activity is very similar in *e.g.* the splanchnic nerves, the renal nerves as well as the cardiac nerves (*e.g.* Downing and Siegel 196[illegible]) is of particular interest in view of the fact that evidence for a discriminative sympathetic nervous control of peripheral organs is accumulating (Folkow 1960)

The afferent activity recorded in this study showed essentially the same pattern as that previously described on the basis of observations made in rats (Åström and Crafoord 1967). It would seem that also in the cat more than one type of mechanoreceptors are involved and that impulses are conducted in fibers of more than one size. Thus under certain conditions *e.g.* mechanical pressure on the hilus area with a glass rod (Fig. 4 D), the response elicited was rapid in onset and consisted of

comparatively large spikes of short duration. In other situations the action potentials appeared to be of somewhat longer duration and of lower amplitude indicating fibers of smaller size (Fig. 6 A).

The response of short latency following elevation of venous pressure or the application of gentle pressure to the hilus area would seem likely to originate from the walls of the large intrarenal veins or their close proximity. The adequate stimulus for this type of mechanoreceptor would seem to be deformation (stretch) of the vessel wall. The second type of response seemed to originate, at least to a large extent, from a different location and was obtained at high perfusion pressures (Fig. 5), prolonged elevation of venous pressure, or close intraarterial injection of saline in the renal artery (Fig. 6). All these procedures have been found to increase the kidney tenseness (Åström and Samelius 1962). The increase in kidney tenseness (intrarenal pressure) would thus seem to be the adequate stimulus for this second type of mechanoreceptors. Ischemia produced a maximum response within a fairly short period of time, 10—15 sec in the experiment shown in Fig. 4 E—H.

The afferent discharge sometimes recorded when the ureter was pinched or compressed with a glass rod, or when the pelvis was distended, seemed to be different from that discussed above. The action potentials were much smaller and broader and probably originated from so-called visceral pain afferents (C-fiber activity).

Several experiments were carried out with the purpose to study the physiological significance of the afferent nerve activity from the kidney. Electrical stimulation of the kidney nerves in the central direction produced no significant changes in systemic blood pressure or heart rate of the anesthetized cat. Nor was gross blood flow, urine formation or sodium excretion of the contralateral kidney affected. Stimulation of one strand of kidney nerves and observation of gross blood flow and sodium secretion in the kidney on that same side, which was thus at least partly innervated, also gave negative results. This would seem to indicate that the afferent information from the kidney subserves some other physiological function than the acute regulation of blood flow in the kidney under resting conditions. In the discussion of the similar results obtained in rats (Åström and Crafoord 1967) it was mentioned that this kind of information from the kidney would theoretically be suitable for the control of the extra vascular fluid volume in a more general way. This possibility remains to be studied.

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References

ADRIAN, E. D., D. W. BRONK and G. PHILLIPS, Discharges in mammalian sympathetic nerves. *J Physiol. (Lond.)* 1932 *74* 115—133

ÅSTRÖM, A. and J. CRAFOORD, Afferent activity recorded in the kidney nerves of rats. *Acta physiol. scand.* 1967 *70* 10—15

ÅSTRÖM, A. and U. SAMELIUS, Tenseness variations in the cat kidney. *Acta physiol. scand.* 1962. *55* 35—44

CASTENFORS, J., Renal function during exercise. *Acta physiol. scand.* 1967 *70* Suppl. 293 1—43

Puromycin was used to block the protein synthesis. This antibiotic is well known to have such an effect on many mammalian tissues (for ref see Darken 1964) and it has earlier been shown in this laboratory that this antibiotic can block the incorporation of labelled amino acids into the protein fraction of the isolated rat ovary (Ahrén and Rubinstein 1965)

In addition to experiments with puromycin and gonadotrophins experiments with incubation of prepubertal rat ovaries in gaseous mixtures containing various proportions of oxygen were performed. It has already been reported that anoxia stimulates glycolysis in both corpus luteum slices and prepubertal rat ovaries (Armstrong 1963, Armstrong et al 1963) but it was considered to be of interest to explore further this "Pasteur" effect in the ovary and particularly whether the gonadotrophins could still stimulate the glycolysis in anaerobiosis. Experiments with addition of 2 4-dinitrophenol to the isolated ovaries will also be reported.

Methods

Animals

Ovaries were obtained from prepubertal 24–26 day old rats of the Sprague-Dawley strain and from rats, hypophysectomized when 25—28 days old and used for experiments 13—15 days later. The completeness of hypophysectomy was checked at autopsy by serial sections through the hypophyseal capsule and adjacent tissues. The rats were maintained on semi-synthetic diet (Gustavsson 1959) and water *ad libitum* at all times. The non-hypophysectomized rats were deprived of food 20—24 hrs before the extirpation of the ovaries.

Chemicals

[illegible] labelled L-leucine-[illegible]H (spec activity 200 μC/μmole) and L-proline-[3]H (spec. activity [illegible]0 μC/μmole) were obtained from the Radiochemical Centre Amersham, England. Puromycin dihydrochloride and the aminonucleoside of puromycin (PAN) were obtained from Nutritional Biochemicals Corp Cleveland USA. 2 4( )-dinitrophenol was purchased from Merck AG Darmstadt Germany. Bovine luteinizing hormone (NIH LH B3) and ovine follicle stimulating hormone (NIH FSH-S2) were supplied by the Endocrinology Study Section of National Institutes of Health, USA. LH was dissolved in 0.9 % NaCl (1 mg/ml) for the injections. Control rats were injected with 0.9 % NaCl and LH and FSH dissolved in Krebs bicarbonate or Tris-HCl buffer for the [illegible] experiments.

R[illegible]moval [illegible] [illegible]batio[illegible] [illegible]

The rats were killed by cervical fracture and the ovaries were rapidly removed and dissected as described previously (Hamberger and Ahrén 1967). The ovaries were collected in groups of [illegible] when both glucose uptake and lactic acid production were measured and in groups of two ovaries when only lactic acid production was determined. The reason for this experimental design is discussed in detail in a previous paper (Hamberger and Ahrén 1967). The ovaries were blotted on filter paper and transferred to 10 ml Erlenmeyer flasks containing 1 ml medium. The flasks contained either Krebs bicarbonate buffer pH 7.4 containing 5.5 mM glucose or Tris-HCl buffer 20 mM pH 7.4 containing 10 mM glucose 5.3 mM KCl 121 mM NaCl 1 mM MgSO[illegible] 1.3 mM CaCl$_2$. When not otherwise stated in Results the flasks were gassed with 95 % O_2 5 % CO_2 or 100 % O_2, respectively and then incubated at 37° C with continuous shaking. Following incubation the media were immediately withdrawn, and the ovaries removed from the flasks rinsed free of adhering incubation medium in ice-cold buffer weighed and homogenized in 10 [illegible] trichloric acid (TCA).

Measurement of glucose uptake and lactic acid production

Ovarian glucose uptake was determined by measuring the initial and final glucose concentrations of the incubation media by the glucose-oxidase method (Saifer and Gerstenfeld 1958). The uptake is expressed as μmoles of glucose taken up per 100 mg of ovarian tissue (wet weight) per hr

Lactic acid production by the ovaries was calculated from the amount of lactic acid found in the medium at the end of the incubation period for reasons discussed elsewhere (Hamberger and Ahrén 1967). Lactic acid was determined by an enzymatic method as described by Lundholm and co-workers (Lundholm, Mohme-Lundholm and Vamos 1963; Lundholm, Mohme-Lundholm and Svedmyr 1963).

Determination of incorporated radioactivity into ovarian protein

After incubation the ovaries were homogenized in 1 ml of 10 % TCA and the precipitate was spun down. The precipitate was washed once in 3 ml of 10 % TCA in excess of unlabelled amino acid and then heated for 15 min at 90° C in 3 ml fresh TCA, spun down again and washed 3 times with ethanol-ether-chloroform (2 : 2 : 1). After the final wash the precipitate was dissolved in 1 ml of 1 N NaOH and boiled for 5 min. The concentration of protein in the NaOH solution was measured according to Lowry *et al.* (1951). The incorporation of radioactivity into the protein was determined by the Schöniger combustion technique (Schöniger 1955) in the following modification. The material (200 µl) was spotted on filter paper with low ash content and allowed to dry before combustion. The combustion vessel (vol. 2000 ml) was gassed with 100 % O_2 for 5 min at room temperature and then sealed. After chilling the vessel for 10 min to around −80° C in a mixture of ethanol and dry-ice combustion was effected. Because of the change in temperature between the gassing period and the combustion, the pressure inside the vessel was considerably lower than the atmospheric pressure surrounding it. It was then possible to introduce 99.5 % ethanol (5 ml) into the vessel to absorb the H_2O without leakage of gas. The vessel was allowed to stand in room temperature for 30 min before scintillation fluid (22 ml toluene containing 0.5 % PPO and 0.02 % POPOP) was introduced. Two 12 ml samples were taken up and counted in a Packard Tri-Carb liquid scintillation spectrometer first without standard and then after addition of a known internal standard. The counting efficiency was between 15—20 %. With this system 95 % (range 93—97 %) of the ³H added as ³H labelled amino acids could be recovered. The radioactivity is expressed as DPM/mg protein.

Measurements of the accumulation of leucine-³H

The amino acid was added to give a final medium concentration of 0.05 mM. After incubation the ovaries were homogenized in 10 % TCA and the radioactivity determined as described earlier (Ahrén and Rubinstein 1965). This amino acid can probably be metabolized to some extent by the ovarian cells and it is therefore likely that some of the radioactivity in the tissue extracts did not represent the original amino acid but rather various metabolites. This has not been analysed in the present study and it is therefore important to point out that the distribution ratios for this amino acid are the ratio between the total intracellular radioactivity and the radioactivity in the incubation medium (= CPM/ml intracellular water : CPM/ml incubation medium).

Statistical procedures

Mean values are given ± standard error of the mean. Comparison between different groups was performed according to Student's *t*-test. A p-value of 0.05 or less was considered significant in this study. Calculations of regression coefficients, tests of linearity and analysis of variance were performed according to Brownlee (1965).

Results

Effects of LH and puromycin in vitro

In the first series of experiments LH (25 µg/ml) was added directly to the incubation medium with or without puromycin (500 µg/ml). The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose and glucose uptake was determined. It can be seen from Fig. 1 that LH administered *in vitro* stimulated the glucose uptake both in the absence and presence of puromycin. Addition of puromycin *in vitro* however decreased the glucose uptake both with and without LH in the medium. In a previous study it was shown that puromycin *in vitro* in this concentration nearly completely blocked the incorporation of labelled amino acids into ovarian protein (Ahrén and Rubinstein 1965). It is, however, clear

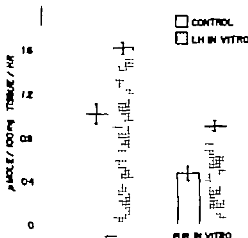


Fig. 1

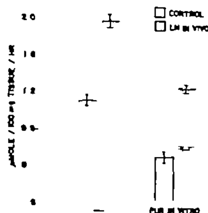


Fig. 2

Fig. 1. Effects of puromycin and LH *in vitro* on glucose uptake by prepubertal rat ovaries. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose. Puromycin (Pur.) was added in a concentration of 500 μg/ml medium and LH in a concentration of 25 μg/ml medium. Each column is the mean of 5 observations and standard errors are indicated by vertical lines on top of the columns. There was a significant effect of LH both in the absence and presence of puromycin ($p<0.001$) and puromycin *per se* caused a significant decrease in glucose uptake ($p<0.001$).

Fig. 2. Effects of puromycin on glucose uptake by isolated ovaries from control rats and rats injected with LH. LH was injected in one i.v. dose (500 μg/100 g b.w.) 4 hrs before removal of the ovaries. Control rats were injected with 0.9 % NaCl. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose. Puromycin was added to the medium in a concentration of 500 μg/ml. Each column is the mean of 5 observations and standard errors are indicated by vertical lines on top of the columns. There was a significant difference in glucose uptake between ovaries from control and LH-injected rats both in the absence and presence of puromycin ($p<0.001$). Puromycin *per se* caused in addition a significant decrease in glucose uptake ($p<0.01$).

Table 1. Influence of puromycin and PAN on the effects of LH on lactic acid production by ovaries from prepubertal rats

Addition to medium	Lactic acid production (μg/100 mg/2 hr) control	LH (25 μg/ml)	Significance of LH effect
—	135.0 ± 4.6 (4)	363.8 ± 21.8 (4)	$p<0.001$
Puromycin (500 μg/ml)	62.2 ± 7.2 (4)	126.3 ± 10.1 (4)	$p<0.005$
PAN (500 μg/ml)	121.7 ± 9.6 (4)	277.8 ± 19.9 (4)	$p<0.005$

The ovaries were preincubated for 30 min in Krebs bicarbonate buffer containing 5.5 mM glucose with and without puromycin and aminonucleoside of puromycin (PAN) respectively. The ovaries were then transferred to new flasks and incubated for 2 hrs in Krebs bicarbonate buffer containing the compounds indicated in the table. Lactic acid was determined in the medium at the end of the incubation period as described earlier (*Hamberger and Ahrén* 1967). Mean values ± S.E. Number of observations in parentheses.

TABLE II Effect of puromycin on the incorporation of proline-^{3}H into protein of ovaries from prepubertal rats.

Addition to medium	control	LH (25 μg/ml)
	644.2±41.3 (5)	687 7±37 6 (4)
Puromycin (500 μg/ml)	11 6± 4.3 (4)	12.9± 3.9 (4)

The ovaries were preincubated for 30 min in Krebs bicarbonat buffer in the absence or presence of puromycin (500 μg/ml) The ovaries were then transferred to new flasks and incubated for 2 hrs in fresh buffer containing the respective compounds indicated in the table. The incorporation of proline ^{3}H is expressed as DPM/μg protein. Mean values±S.E. Number of observations in parenthesis.

that the effects of puromycin are not immediate, and a limited synthesis of proteins at the beginning of the incubation period can therefore not be excluded. In the following experiments, the ovaries were therefore preincubated with puromycin in the medium for 30 min in order to create a complete blockage of the protein synthesis before LH was added. In these experiments lactic acid production was measured instead of glucose uptake, since t has been shown that the effects by LH are very similar on these two parameters in the prepubertal ovaries (Hamberger and Ahrén 1967) After the preincubation period, the ovaries were transferred to new flasks and incubated for 2 hrs in Krebs bicarbonate buffer according to the scheme in Table I L-proline-^{3}H was added to all the flasks in a concentration of 0.1 mM. It can be seen from Table I that there was a highly significant stimulation of lactic acid production by LH both in the absence and presence of puromycin. However in both the control and LH group puromycin decreased the level of lactic acid production by 50 % The incorporation of proline into ovarian protein was totally blocked by puromycin in these experiments (Table II)

Even if the main result of the above mentioned experimen ts showed that puromycin did not block the effect of LH, another interesting observation was that this antibiotic decreased the rate of glycolysis in the isolated ovaries both in the presence and absence of LH in the incubation medium. In order to explore whether this ef fect of puromycin on the ovarian carbohydrate metabolism might be related to its effects upon the protein synthesis, other groups of ovaries were incubated in th presence of the aminonucleoside of puromycin (PAN) It has been shown that this compound, which does not block the protein synthesis, exerts many of the "unspecific effects of puromycin which are not related to a blockage of the protein synthesis (Hofert *et al.* 1962, Hofert and Boutwell 1963 1966, Korner nd Raben 1964) It can be seen from Table I that PAN did not significantly nfluence he lactic acid prod ction by the ovaries.

It has recently been reported (Hamberger and Ahrén 196 that LH *tro* stimulates lactic acid production also in ovaries from hypophysec mized rats T ble III shows results from experiments where such ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose and 0.05 mM L-leucine-^{3}H

T ABLE III Effects of puromycin and LH on lactic acid production and on intracellular accumulation and incorporation of L-leucine H by ovaries from hypophysectomized rats.

Addition to medium	Lactic acid µg/100 mg × 2 hr	Leucine- H accumulation	Leucine-³H incorporation DPM/µg protein
—	295.7 ± 11 9	2.29 ± 0 15	2007.8 ± 78.2
LH	64 7 ± 43.4	2 78 ± 0.12	2035.1 ± 184 8
Pyromycin	115.3 ± 14 0	7.87 ± 0.60	26.4 ± 3.9
LH + pyromycin	326.3 ± 53.8	7 13 ± 0.44	28.4 ± 3.9

The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 0.05 mM L-leucine-H and 5.5 mM glucose LH was added to the medium in concentration of 25 µg/ml and puromycin in concentration of 500 µg/ml. The intracellular accumulation of leucine H is expressed as distribution ratio (CPM/ml medium CPM/ml intracellular H_2O) There re 3 bservations in each group and the results are given as mea ± S.E.

The same pattern as for the prepubertal rats was found i.e a stimulation of lactic acid production by LH both in the absence and presence of puromycin and a significant decrease in the rate of lactate production by puromycin. It can also be seen from Table III that there was a marked increase in the intracellular accumulation of leucine in the presence of puromycin due to the almost completely blocked amino acid incorporation into protein.

Eff cts of puromycin and LH in vivo

Fig 2 shows results from experiments where LH was injected to 4—26 day old rats in one i.v dose (500 µg 100 g b.w) 4 hrs before removal of the ovaries. The ovaries were then incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose with and without addition of puromycin (500 µg ml) *In vivo* administration of LH significantly stimulated the glucose uptake both in the presence and absence of puromycin. As in the above mentioned experiments, however puromycin decreased the rate of glucose uptake in ovaries from both control and LH injected rats.

In one series of experiments prepubertal rats were anesthetized with nembutal. Fortyfive min before removal of the ovaries puromycin (5 mg) was injected to the animals i.p. and 15 min after this injection they were given one i.v injection of LH (500 µg 100 g b w) The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5 5 mM glucose and lactic acid production was measured at the end of the incubation period. Even under these experimental conditions there was a significant stimulation by LH ($p < 0.005$) both in the absence and presence of puromycin (Fig 3)

Effects of puromycin and FSH i t o

It has been shown earlier that FSH i vit timulates lactat production in prepubertal rat ovaries (Hamberger and Ahrén 1967 Ahren et al. 1968a) and that

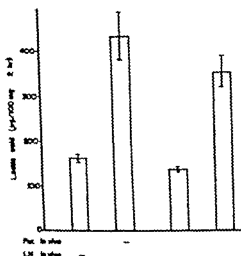


Fig. 3

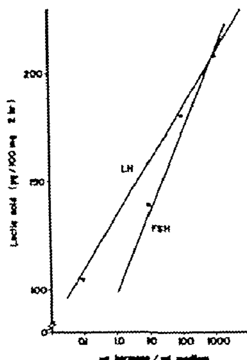


Fig. 4

Fig. 3. Lactic acid production by isolated ovaries from prepubertal rats. All rats were anesthetized with nembutal i.p. (4 mg/100 g b.w.) one hr before the start of incubation. LH was injected i. (500 µg/100 g b.w.) 30 min before removal of the ovaries and puromycin (Pur.) was injected i.p. (5 mg) 15 min before the LH injection. The ovaries were incubated for 2 hrs at 37 C in Krebs bicarbonate buffer containing 5.5 mM glucose. Each column is the mean of 3—4 observations and standard errors are indicated by vertical lines on top of each column. The effect of LH was significant both in ovaries from rats not injected with puromycin ($p<0.003$) and in those from rats injected with puromycin ($p<0.005$).

Fig. 4. Dose-response lines for the effects of NIH LH-B3 and NIH-FSH S2 on lactic acid production in presence of puromycin by ovaries from prepubertal rats. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose and puromycin (500 µg/ml). The regression equations are $Y = 166 + 25$ (x—3.5) and $\hat{Y} = 172 + 38$ (x—5.0). The slopes of the two dose-response lines differ significantly ($p<0.001$).

there was a log linear dose response relationship of this parameter when FSH (NIH FSH-S2) was added to the incubation medium in concentrations varying between 10—1000 µg ml. This effect by FSH could not be explained as due to a contamination of LH in the FSH preparation since there was a significant difference in the slopes of the dose response curves for LH and FSH. One series of experiments was now undertaken in order to investigate whether this effect by FSH could be blocked by puromycin. The ovaries were incubated in Krebs bicarbonate buffer containing 5.5 mM glucose and puromycin in a concentration of 500 µg ml. FSH was added in concentrations varying between 10—1000 µg ml. For comparison LH was added to other groups in concentrations varying between 0.10—1000 µg ml. Lactic acid production was measured and the results are summarized in Fig. 4. Both FSH and LH

TABLE IV Effects of LH on glucose uptak and lactic cid production by prepubertal rat ovaries in various gaseous mixtures.

	Number of expts.	Control	LH (100 µg/ml)	Stimulation by LH in per cent of control	Significance of LH effect
Glucose uptake					
100 O	4	1.20 ± 0.14	2.06 ± 0.25	72	$p<0.05$
60 O – 40 N	5	1.61 ± 0.10	2.01 ± 0.07	25	$p<0.05$
100 N	4	1.81 ± 0.03	1.85 ± 0.11	2	N.S.
Lactic acid production					
100 O_2	5	0.94 ± 0.19	–.01 ± 0.38	114	$p<0.01$
60 O_2 – 40 N	5	2.01 ± 0.13	2.31 ± 0.17	15	N.S.
100 N_2	4	–.71 ± 0.19	2.74 ± 0.06	1	N.S.

The ovaries wer incubated for 2 hrs in Tris-HCl buffer containing 10 mM glucose in arious gaseous mixtures indicated in the table nd glucose uptake and lactic acid production were measur ed at the end of the incubation period.

Glucose uptake nd lactic acid production are expressed as µmole/100 mg × hr Mean values ± S.E. N.S. = not significant.

could stimulat lactic acid production in the presence of puromycin and there was a log linear dose response relationship for the two hormones with a significant dif ference in slope ($p<0.001$) Although the rate of lactic acid production was considerabl reduced by puromycin (30 %) the sensitivity for the respective hormones did not seem to be affected by the antibiotic

Eff t of ano ia nd 2,4-din t ophenol

In the first series of experiments, ovaries from prepubertal rats were incubated for 2 hrs in Tris-HCl buffer containing 10 mM glucose LH was added to the medium in a concentration of 1000 µg ml ince this dose has been shown to give maximal effects upon the parameters tudied The flasks were gased with 100 % O_2, 60 % O + 40 % N_2 and 100 N respectively Glucose uptake and lactic acid production were measured nd the results are ummarized in Table IV There was a significant stimulatory effect of LH n glucose uptake and lactic acid production in 100 O and of glucose uptak n 60 O – 40 % N_2 while there were no significant ffects by the hormone in anaerobic milieu From Table IV it can be seen that ther is a gradual increase in th ntrol alues with a decreasing oxygen tension.

The effect of puromycin n lactic acid production by ovaries incubated in anaerobic milieu wa alw tudied The ovaries were then incubated for 2 hrs in Krebs bicarbonate buffer contain ng 5.5 mM glucose in flasks gased w th 95 N + 5 CO It was found that purom n 500 µg ml significantl decreased lactic

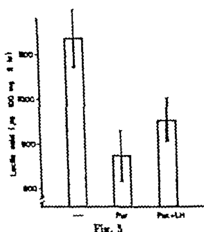


Fig. 5

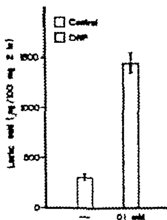


Fig. 6

Fig. 5 Lactic acid production by anaerobically incubated ovaries from hypophysectomized rats. The ovaries were incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose. The flasks were gassed with 95 % N_2 + 5 % CO_2. Addition of puromycin (500 µg/ml) significantly decreased the lactate production ($p<0.02$) while no significant stimulatory effect was elicited by LH (1000 µg/ml) in the presence of puromycin under these experimental conditions. There wer 3—4 ovaries in each group and the standard errors ar indicated on top of the columns.

Fig. 6. Effects of 2,4-dinitrophenol (DNP) on lactic acid production by ovaries from hypophysectomized rats. The ovaries wer incubated for 2 hrs in Krebs bicarbonate buffer containing 5.5 mM glucose and DNP in concentration of 0.1 mM. The effect of DNP is highly significant ($p<0.001$). Standard errors are indicated on top of the columns.

acid production by these ovaries ($p<0.02$). LH, however did not stimulate the lactic acid production by these ovaries (Fig. 5).

In another series of experiments, ovaries from hypophysectomized rats were incubated in Krebs bicarbonate buffer containing 5.5 mM glucose and dinitrophenol (DNP) in concentrations varying between 0.1—5 mM. The flasks were gassed with 95 % O_2 + 5 % CO_2 and lactic acid production measured. *Already the lowest concentration of DNP* gave a maximal stimulation of lactic acid production by the ovaries (Fig. 6).

Discussion

The results of the present study show that both LH and FSH can still increase glucose uptake and lactic acid production in the prepubertal rat ovary when the protein synthesis is blocked by the antibiotic puromycin. It is therefore not likely that the synthesis of new protein is involved in the cellular reactions leading to increased glycolysis after administration of these gonadotrophin. The same type of experimental results and the same type of interpretation of these results have been made in studies concerning the regulation of glucose uptake in the mammalian muscle tissue. In this tissue e.g. in the rat diaphragm, insulin stimulates the rate of uptake of both glucose and certain non-metabolizable sugars and the effect of insulin cannot be

blocked by puromycin (e g Eboué Bonis et al. 1963 Sovik 1965) Furthermore it has been shown that pituitary growth hormone can stimulate the rate of uptake of monosaccharides in the isolated rat diaphragm under certain experimental conditions, and that this effect of growth hormone cannot be blocked by puromycin (Hjalmarson 1968) The fact that LH and FSH still showed different slopes of the dose-response curves when the ovaries were incubated with various concentrations of these hormones in combination with puromycin, confirm our previous conclusion (Hamberger and Ahrén 1967 Ahrén et al 1968a) that the effect of FSH on ovarian glycolysis cannot be explained as due to a contamination of LH in the FSH preparation. The finding that FSH still stimulated the ovarian carbohydrate metabolism when puromycin was present is in addition of interest in relation to the recent observation that the effect of this gonadotrophic hormone on amino acid transport in the isolated ovary is blocked by this antibiotic (Ahrén, Hamberger and Hartford 1967) Another difference between the effect of FSH on ovarian amino acid transport on the one hand and on ovarian carbohydrate metabolism on the other is that the former effect is seen only after *in vivo* administration of the hormone while the latter effect is seen both after *in vivo* and *in vitro* administration. The result of the present study is therefore further evidence in favour of the hypothesis that FSH has two separate effects on the metabolism of the rat ovary one on amino acid transport and protein synthesis and the other on ovarian carbohydrate metabolism.

Armstrong, Kilpatrick and Greep (1963) reported that the rates of glucose uptake and lactic acid production by the isolated prepubertal rat ovary were increased by anoxia and this observation has been confirmed in the present study They also reported that addition of LH to the anaerobically incubated ovaries did not further increase ovarian glycolysis. Armstrong and co-workers used medium concentrations of LH betwen 10 and 25 µg ml in these experiments and it has subsequently been shown (Hamberger and Ahrén 1967) from this laboratory that these concentrations of LH did not produce a maximal glycolysis in the isolated ovaries. It could therefore not be concluded from the experiments by Armstrong et al. (1963) whether the anaerobicall incubated ovaries were completely insensitive to LH. However n the present tudy it wa hown that not even a very high medium concentration of LH (1000 µg ml) could give a further timulation of glucose uptake and lactic acid production by the isolated ovaries incubated under anaerobic conditions. Under the present experimental conditions a maximal rate of glycolysis in the isolated ovaries seems therefore to exist which can be reached under anaerobical conditions without any hormone in the medium and under aerobic conditions in the presence of a high concentration of LH These observations are compatible with the theory that the cellular mechanisms leading to an increased glycolysis under anaerobic conditions might be th same as those leading to increased glycolysis after administration of LH Many theories ha e been proposed for the mechanism of the Pasteur effect but the cellular mechanism for this effect is till not f lly understood. It seems, however quit clear that changes in the intracellular levels of inorganic phosphate (P_i) and adenine nucleotides are of importance for this effect (g

Lynen 1957 Gatt and Racker 1959 Morgan, Randle and Regen 1959) A more precise theory which during recent years has been supported by many observations is that the immediate reason for the increased rate of glycolysis under anoxia is an increase in the activity of the phosphofructokinase enzyme system (PFK) (e g Newsholme and Randle 1961 Passonneau and Lowry 1962, Uyeda and Racker 1965) This increase in PFK activity is in turn produced by a decrease of ATP in combination with an increase in P_i and ADP changes which are consequences of the lack of oxygen. The recent observation (Ahrén *et al* 1968b) that addition of LH to isolated prepubertal rat ovaries significantly decreased the ATP level favours therefore the above mentioned theory that the cellular mechanism of glycolysis activation by LH might be the same as that of anoxia.

Although the main result of the puromycin experiments was that the gonadotrophins could still stimulate ovarian glycolysis in the presence of this antibiotic, another result of these experiments was as striking, namely that puromycin markedly decreased the rates of glucose uptake and lactic acid production under all experimental conditions explored i.e under aerobic and anaerobic conditions as well as with and without gonadotrophins present. The mechanism for this inhibitory effect of puromycin on ovarian carbohydrate metabolism is not clear but there are two implications either that this effect of the antibiotic is related to its inhibitory effect on protein biosynthesis, or that it is a more direct effect on some step(s) of the carbohydrate metabolism. Such direct effects on the carbohydrate metabolism hav been reported by Hofert and co-workers (Hofert *et al.* 1962, Hofert and Boutwell 1963 1966) who found that puromycin induced glycogenolysis as an event independent of inhibited protein synthesis in mouse liver and Korner and Raben (1964) who found that the insulin effect on epinephrine stimulation of fatty acid release from adipose tissue was inhibited by puromycin by virtue of its amino nucleoside-like properties and not as a consequence of its effect on protein synthesis. In these last mentioned studies various puromycin analogues had effects similar to puromycin on the parameters studied without appreciable effect on protein synthesis. The fact that the amino nucleoside of puromycin (PAN) had no significant effects on glucose uptake and lactic acid production in the ovaries strongly indicates that the effect of puromycin on these parameters is linked to its effect on protein synthesis. Further in an anaerobic milieu puromycin still produced a significant decrease in lactate production. This precludes the possibility that puromycin might exert its effects by influence on mitochondrial activity

In connection with this effect of puromycin on ovarian carbohydrate metabolism it is of interest to note that, in isolated mammalian muscle preparations with intact cell membranes puromycin decreases the rat of uptake not only of glucose but also of nonutilizable monosaccharides (e g Hjalmarson 1968) thus indicating an influence of puromycin on the membrane transport of these substances Moreover in both isolated rat ovaries (Ahrén and Rubinstein 1965) and in isolated muscle preparations (Kostyo and Redmond 1966 Arvill and Ahrén 1967 Hjalmarson 1968) puromycin has an inhibitory effect also on the rate of uptake of amino acids i.e

on another carrier mediated transport system in the cell membranes. Kostyo and Redmond (1966) suggested from their studies on amino acid transport in the isolated rat diaphragm that puromycin suppresses the amino acid uptake by interfering with the synthesis of proteins involved in the transport process. As a consequence of this comparison with the effect of puromycin on the metabolism of the muscle tissue, we propose that the inhibitory effect of puromycin on ovarian glycolysis is produced at least in part, on the level of glucose uptake by the cells and that the mechanism for this effect on the membrane transport might be the same as that suggested by Kostyo and Redmond (1966)

Studies are now in progress in order to investigate whether puromycin suppresses the rate of uptake of nonutilizable monosaccharides in the isolated rat ovary

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References

AHRÉN, K. and J. L. KOSTYO: Acute effects of pituitary gonadotrophins on the metabolism of isolated rat ovaries. *Endocrinology* 1963. *73*. 81—91.

AHRÉN, K. and LISE RUBINSTEIN: Effects of follicle stimulating hormone on amino acid transport and protein biosynthesis in the isolated rat ovary. *Acta physiol. scand.* 1965. *64*. 463—474.

AHRÉN, K., L. HAMBERGER and MARIA DE HART ORD: Further studies on the effect of follicle stimulating hormone on amino acid transport in the isolated rat ovary. *Acta physiol. scand.* 1967. *71*. 11—273.

AHRÉN, K., E. B., L. A. HAMBERGER and T. PERKLEV: Effects of human menopausal gonadotropin and ovine follicle stimulating hormone on lactic acid production by ovaries from prepubertal rats. *Endocrinology* 1968a. *82*. 420—422.

AHRÉN, K., E. B. A. BENGTSSON, L. A. HAMBERGER and L. LUNDHOLM: Mechanisms for the action of luteinizing hormone (LH) on the carbohydrate metabolism of the prepubertal rat ovary. *Acta physiol. scand.* 1968b. *73*. 23A.

ARMSTRONG, D. T.: Stimulation of glycolytic activity of rat corpus luteum tissue by luteinizing hormone. *Endocrinology* 1963. *72*. 908—913.

ARMSTRONG, D. T., R. KILPATRICK and R. O. GREEP: In vitro and in vivo stimulation of glycolysis in prepubertal rat ovary by luteinizing hormone. *Endocrinology* 1963. *73*. 165—169.

ARVILL, A. and K. AHRÉN: Effects of insulin on the intact levator ani muscle of the rat. *Acta endocr. (Kbh.)* 1967. *56*. 295—307.

BROWNLEE, K. A.: *Statistical theory and methodology*. ed. John Wiley & Sons Inc. New York 2 ed. 1965.

DARKEN, M. A.: Puromycin inhibition of protein synthesis. *Pharmacol. Rev.* 1964. *16*. 223—243.

EBOUÉ-BONIS, D., A. M. CHAMBAUT, P. VOLFIN and H. CLAUSER: Action of insulin on the isolated rat diaphragm in the presence of actinomycin D and puromycin. *Nature (Lond.)* 1963. *199*. 1183—1184.

GATT, S. and E. RACKER: Regulatory mechanisms in carbohydrate metabolism II. *J. biol. Chem.* 1959. *234*. 1024—1028.

GUSTAFSSON, B. E.: Lightweight stainless steel systems for rearing germfree rats. *Ann. N. Y. Acad. Sci.* 1959. *78*. 1.

HAMBERGER, L. and K. AHRÉN: Effect of gonadotrophins in vitro on glucose uptake and lactic acid production of ovaries from prepubertal and hypophysectomized rats. *Endocrinology* 1967. *81*. 93—100.

Acta physiol. scand. 1968. 74. 91—95

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A "Two-Compartment Diver" in the Micro-Diver Technique

By

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Abstract

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A technique is described which makes it possible and easy to mix solutions in the micro-diver at any time during an experiment. The mixing procedure is completed within 5 min and does not in itself cause any alterations in the rate of gas consumption. This two-compartment diver can be used for measurements of gas consumptions in the order of 10 µl/hr thus allowing determinations on single cells. Some results from experiments on isolated granulosa cells from the rat ovary are reported to illustrate the usefulness of the technique.

The use of Cartesian divers for microgasometric measurements was introduced by Linderstrøm-Lang in 1937. Initially analyses were made of serum samples and of tissue fragments (e.g. Boell and Shen 1950) and later of numerous biochemical and certain inorganic reactions (e.g. Holter and Løvtrup 1949). The technique is suitable for measurements of gas consumption in the order of 10 —10 µl/hr. Various methods for initiation of reactants and inhibitors to the metabolism of the tissue tested in this type of diver has also been described (Claff and Tahmisian 1949, Hellerström 1967).

An even more sensitive method, applying the Cartesian diver principle, was introduced by Zeuthen in 1953. This ampulla or micro-diver respirometer measures gas consumption or production in the order of 10 µl/hr, thus allowing determinations on single cells. Various methods for addition of reactants and inhibitors have also been reported for this technique. The micro-diver can be removed from its flotation vessel in the thermostatically controlled bath, then opened, and the incubation medium replaced by a fortified medium (Zajicek 1957, Brzin and Zeuthen 1961) or the reactant in question can be drawn up into the micro-diver and mixed with the initial incubation medium (Giacobini 1959, 1962). The micro-diver is then sealed and replaced in the flotation vessel and after a temperature equilibration period of approximately 30 min the effect of the added reactant can be recorded. This procedure requires manipulation of the diver during the experiment and it can therefore in itself influence the reaction studied in the diver.

on another carrier-mediated transport system in the cell membrane. Kostyo and Redmond (1966) suggested from their studies on amino acid transport in the isolated rat diaphragm that puromycin suppresses the amino acid uptake by interfering with the synthesis of proteins involved in the transport process. As a consequence of this comparison with the effect of puromycin on the metabolism of the muscle tissue, we propose that the inhibitory effect of puromycin on ovarian glycolysis is produced, at least in part, on the level of glucose uptake by the cells and that the mechanism for this effect on the membrane transport might be the same as that suggested by Kostyo and Redmond (1966).

Studies are now in progress in order to investigate whether puromycin suppresses the rate of uptake of non-utilizable monosaccharides in the isolated rat ovary.

We wish to thank the Endocrinology Study Section of NIH for the generous supply of LH (NIH-LH-B3) and FSH (NIH-FSH-S[illegible]). [illegible] of the puromycin for the experiments [illegible] was a gift from Ferrosan Ltd, Malmö, Sweden.

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References

Ahrén, K. and [illegible]: Acute effects of pituitary somatotrophin on the metabolism of isolated rat ovaries. *Endocrinology* [illegible].

Ahrén, K. and [illegible]: Effects of follicle stimulating hormone on amino acid transport and protein synthesis in the isolated rat ovary. *Acta physiol. scand.* 1965, 64, [illegible].

Ahrén, K., L. Hamberger and [illegible] Hamberger: Further studies on the effect of follicle stimulating hormone on amino acid transport in the isolated rat ovary. *Acta physiol. scand.* [illegible].

Ahrén, K., L. B. [illegible], L. A. H[illegible] and T. [illegible]: Effects of human menopausal gonadotrophin [illegible] hormone on lactic acid production by ovaries from [illegible] rats. *Endocrinology* [illegible] 82, [illegible].

Ahrén, K., L. B. [illegible], L. A. [illegible] and L. [illegible]: Mechanisms for the action of luteinizing hormone (LH) on the carbohydrate metabolism of the [illegible] ovary [illegible].

Armstrong, D. T.: [illegible] activity of rat corpora lutea [illegible] by luteinizing hormone. *Endocrinology* [illegible].

Armstrong, D. T., [illegible] and R. O. Greep: [illegible] by luteinizing hormone. *Endocrinology* 1963, [illegible].

[illegible]: Effects of insulin on the [illegible] of the rat diaphragm [illegible].

[illegible], K. A.: [illegible] ed. John Wiley & Sons Inc., New York [illegible].

[illegible], M. A.: [illegible] of protein synthesis. *Pharmacol. Rev.* [illegible].

Eboué-Bonis, D., A. M. Chambaut, P. Volfin and H. Clauser: Action of insulin on the isolated rat diaphragm in the presence of actinomycin D and puromycin. *Nature (Lond.)* 1963, 199, 1183.

Gatt, S. and E. Racker: Regulatory mechanisms in carbohydrate metabolism. II. *J. biol. Chem.* [illegible].

[illegible], B. L.: [illegible] *Ann. N.Y. Acad. Sci.* [illegible].

Hamberger, L. and K. Ahrén: Effects of gonadotrophins in vitro on glucose uptake and lactic acid production of ovaries from [illegible] and hypophysectomized rats. *Endocrinology* 1967, 81, [illegible].

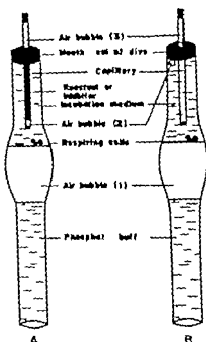


Fig. 1. Schematic drawing of the two-compartment diver. Dimensions: length between 20 and 25 mm, diameter 0.3—0.5 mm. The micro-diver is filled in its upper part with incubation medium containing cells and in its lower part with phosphate buffer. A small capillary (diameter approx. 70 μ) containing the reactant or inhibitor, the effect of which is to be studied, is introduced into the upper end of the diver and fixed by beeswax (mouth seal of diver). The small capillary is sealed in its upper end by beeswax, and in both ends there are air bubbles. Air bubble 2 serves as lock and prevents the content of the capillary to mix with the incubation medium (A). After determination of the control respiratory rate, the pressure around the diver is decreased. This leads to an expansion of air bubble 3 which thus pushes away air bubble 2, allowing the reactant or inhibitor to reach the cells (B). The sizes of air bubble 2 and 3 should be in the ratio 1 : 4 in order to avoid drastic alterations of the pressure.

capillary. After a temperature equilibration period of 30 min the oxygen uptake is estimated from repeated measurements of the equilibrium pressure every 5—10 min for around one hr (Fig. 2).

Addition of reactants or inhibitors

After the measurements of the "control" respiratory rate of the cell(s) the pressure in the flotation vessel is decreased by means of manometers. Air bubble 3 (Fig. 1) in the upper end of the capillary expands due to the decreased pressure in the flotation vessel and consequently air bubble 2 in the lower end of the capillary is pushed away, thus allowing the content of the capillary to mix with the incubation medium. By alternately decreasing and increasing the pressure in the manometric system several times the mixing process is promoted. Changes in pressure can, however, affect the respiratory rate *per se* (Zaijicek and Zeuthen 1961). This influence has been investigated in the present study and very small changes in the slopes of the curves occurred for "control divers" (see Fig. 2) when the maximal change in manometric pressure did not exceed 100 mmHg. In ten control divers a maximal decrease in respiration of 8 % was recorded after the mixing (mean −3.5 %). No control divers showed increased respiratory rate after the mixing procedure. The addition and mixing procedures do not take more than 2—3 min after which immediate registrations of the effect induced by the reactant can be recorded. The effect of the agent introduced can often be registerd after only 10—15 min (Fig. 2).

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Pulse and Respiratory Grouping of Sympathetic Impulses in Human Muscle Nerves

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Abstract

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A haracteristic type of spontaneous activity consisting of grouped discharges of impulses was detected when recording with a tungsten semi-microelectrode from peripheral nerv bundles innerv ting human sk letal muscles. An analysis of this phenomenon shows that the grouped discharges are due to olley of impulses n eff rent nerv fibres which are not skeletomotor Some findings suggest that the efferent conduction locity is ery low (about 1 m/sec) The bursts were often pulse-synchronous and they tended to appear periodically during certain phases of he respiratory cycle. They were not accompanied by any significant changes i the intensity of the afferent responses to phasic or sustained muscle stretch. I some experiments, epeated noxious stimuli triggered showers of impulses in the muscle nerves, simila to the ntaneous bursts. The findings lead to the conclusion that the phenomenon is due to r thmic outbursts of impulses in sympathetic nerv fibres.

Recent reports show how impulses from cutaneous or muscular mechanoreceptors can be recorded in man with a tungsten electrode inserted percutaneously into a peripheral nerve (Hagbarth and Vallbo 1967 a, Hagbarth and Vallbo 1967 b Vallbo and Hagbarth 1968) In these studies, spontaneous discharges of afferent impulses were sometimes seen, both in cutaneous and muscular nerve bundles. Atten tion was soon focused upon characteristic type of "spontaneous" activity which was encountered preferentially in muscle nerves and which did not appear to be of peripheral origin. It consisted of grouped discharges of impulses, repeated either at irregular interval or in a rhythmical fashion bursts occurring at a frequency of about 1/sec often appeared in series alternating with periods of relative silence An analysis of this phenomenon is presented in the following

Methods

All experiments were performed on o healthy male adults (the authors) Recordi gs from more than 200 needle positions were made in the following nerves, the peroneal nerve at knee joint le l, the tibial nerv in the popliteal fossa, the ulna nerv t the lbow the median nerve about 5 cm proximal to the lbow and the superficial branch of the radial nerv about cm proximal to the rist

Record g d stimulatio The electrodes used for nerv recording were coated tungsten needles with diameter of 200 μ and an uninsulated tip with diameter of 5—15 μ. Thei mpedance tested at 1000 H was 10—50 kohm. Such needle soldered to thi copper ire was inserted manually through the skin into the nerv trunk to be explored. No extra fixation of the needle was used. A similar needle bared of insulation for about 5 mm t its end, served as reference. It was placed subcutaneously within 1—3 cm from the nerv elec trode. Details with respect to recording and display systems are given in another paper (Vallbo and Hagbarth 1968)

EMG was recorded either with surface lectrodes (Disa 13 K 62) or with pair of partially coated needles, more or less widely separated within the muscle I order to detect even ery eak muscle contractions high gai was used and the most adequate lectrode positions were tested out before each experiment started. A pai of surface electrodes placed to the left of sternum in the third intercostal space were used to record the ECG and the EMG of inspiratory muscles. ECG and EMG signals were afterwards separated by means of RC-filters before being displayed on an oscilloscope

The tibial and peroneal nerves were examined with the subject lying on table in comfortable prone position. The foot extended over the edge of the table where it was attached to plate turnable around an axis corresponding to that of the pper ankle joint. The rotations of the plate were sensed by potentiometer providing continuous analogue signal of the dorso-ventral position of the foot. The foot plate could also be locked in any position and then strain gauge, attached t the toe-end of the plate, served to measure the activ force exerted by the subject during voluntary contractions of the foot flexors and extensors When the nerves in the upper extremity were examined the subject sat with the elbow and fore-arm supported in about 90 flexion care being taken t make the position as stable and comfortable as possible. Local mechanical stimuli were pplied manually with the flat end of short rod (diameter 4 mm) It was attached to strain gauge sensing the force of the pressure pplied Noxious electric stimuli were obtained from stimulator (Disa 13 G04) delivering rectangular oltage pulses of 1 msec duration and about 150 V amplitude t surface electrodes overlying the ulnar nerv at the lbow

Tw double beam oscilloscopes were used for visualizing the neural and muscular signals as well as the signals from the mechanical transducers. All theses events were also recorded on four channel tape recorder (Precision, type 6204) and after each experiment they were reproduced on an oscilloscope and photographed on running 35 mm paper with Grass camera The nerve signals reproduced were occasionally rectified and "integrated" by low-pass filter with time constant of 15 msec

The test used t discrim at between cutan ous and muscular erve b ndles has been described in detail elsewhere (Vallbo and Hagbarth 1968 Hagbarth and Vallbo 1968) Usually cutaneous nerv bundles were easily identified by the temporary superficial paraesthesias which occurred upon electrode insertion and by the fact that neural discharges ppeared when light mechanical stimuli were applied to the skin area where the paraesthesias were fel Muscle nerv bundles could in similar way be identified by the cramp-like dull paraesthesias which upon electrode insertion occurred in some muscle innervated by the nerve, and by the fact tha neural discharges appeared when this muscle was mechanically ffected either by local stimuli or by muscle stretch or contraction

In our experience, the needle insertions into the nerves hav no serious traumatic effects Moderate temporary paraesthesias occurred occasionally during one or two weeks following an experiment, but no enduring symptoms of neuropathy hav been noted during the two years of experimentation. Clinical EMG-examinations hav revealed no signs of denerv tion

Results

General characterist c of the grouped discharg The intermittent spontaneous bursts of impulses, mentioned in the introduction ppeared more or less distinctly in practically all recordings from muscle nerve bundles, whereas they were never clearly seen when recording from cutaneous nerves In some recordings from the mixed peroneal nd tibial nerves spontaneous bursts intermingled with afferent signals of cutaneous origin but in such cases t could not be excluded that both muscular and cutaneous nerve fibres lay close to the electrode tip. Fig 1 A shows a recording from a pure cutaneous nerve. There wer no spo taneous bursts, merely

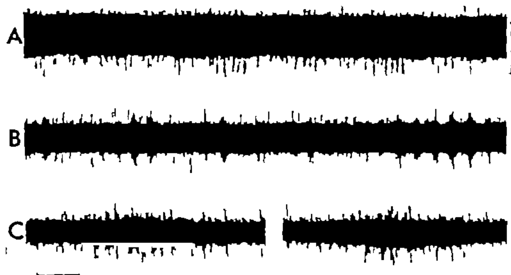


Fig 1 A Spontaneous, sustained discharge of impulses in the superficial branch of the radial nerv B. Upper trace sustained discharge of discrete afferent impulses (positiv spikes) presumabl from muscle stretch receptors (f Fig 3) intermingling with series of grouped discharges in a tibial nerv bundle supplying the medial gastrocnemius muscle Lower trace no signs of motor unit activ ty detected in EMG recording from medial gastrocnemius muscle (needle electrodes

C Grouped discharges on expanded time scale Time in A and B 2 sec i C 0 1 sec Calibration for nerv record 20 V Downward deflections indicate negativity at the intraneural lect ode in this and all succeeding figures.

sustained discharge of impulses, a type of pontaneous activity from skin receptors which has been described in a separate paper (Vallbo and Hagbarth 1968 For mparison, a tracing obtained from a muscle nerv bundle in the tibial nerve is hown in Fig 1 B On insertion, deep paraesthesias occurred in the upper part of the medial ga trocnemius muscle accompanied by a short injury discharge As long a the electrod remained in this recording position (about 30 min) two distinctly different types of spontaneous acti ity were seen (i) a sustained acti ity con isting of repetetiv positive pikes which, as revealed by peripheral tests (*cf* Fig 3 were afferent ignals originating from mechanoreceptors in the calf muscles, (ii) series of grouped discharges of impulses appearing in a rhythmical fashion during th whole recording apparentl uninfl enced by peripheral mechanical stimuli. Th bursts had a d ration of about 0 5 sec nd they were ften spindle-shaped with a gradual onset and decline E en on expanded time scal as in Fig 1 C, the individual impulses constituting the burst wer hard t discriminate partly because they had an mplitude which barel exceeded th noise level of the recording sy tem (peak to-peak 5—10 μV) The peak t peak amplitude of the grouped discharges seldom exceeded 20 μV Usually the negat downward deflections dominated and in some records the bursts seemed to consist almost exclusively of negative spikes (see Fig 6 nd 7)

Sometimes, the burst ppe red in a rather irregular fashion but at other times



Fig. 2. *A* Spontaneous grouped discharges in the tibial nerve. *B*. I jury discharge appearing upon movement of the needle within the nerv . *C*. Spontaneous high frequency discharge with sudden onset (injury?) appearing in the tibial nerve. 5.5 sec are omitted where the trace is interrupted. Time 0.4 sec. Calibration 20 µV

they were repeated rhythmically with a predominant frequency of about 1/sec. Such rhythmical bursts often appeared in short series, interrupted by pauses of variable duration. Occasionally sequences of 5—10 bursts alternated with about equally long periods of relative silence. Such marked regularity however seldom persisted ery long sooner or later t was interrupted or replaced by more irregular patterns.

The source f the dis h rg An initial notion that the bursts might be electrode artefacts or injury discharges had soon to be abandoned. This notion was discred ted already by the difference observed between skin- and muscle nerves and by the fact that bursts with the characteristics described never occurred when the electrode was placed in the tissues surrounding the nerve Neither did they occur in response to deliberate movements of the needle within the nerve. In Fig 2, the spontaneous grouped discharges (A) may be compared with typical injury bursts induced by movements of the needle (B) and a kind of activity consisting of high frequency discharges with sudden onset (C) The latter type of activity which was seen only rarely might be related to some unintentional movement of the needle. The injury discharges were readily discriminated from the intermittent bursts, partly because the injury spikes were always positi e in contrast to those embodied in th bursts. In contrast to most injury discharges, the intermittent bursts were not accompanied by paraesthesias or any other kind of sensory experience

It was easy to rule out skeletomotor nerve fibres as th source of the burst impulses. No muscle contractions accompanied the neural events described and simultaneous EMG-recordings confirmed that in the muscles pplied no motor nits were active during the bursts (see Fig 1 B)

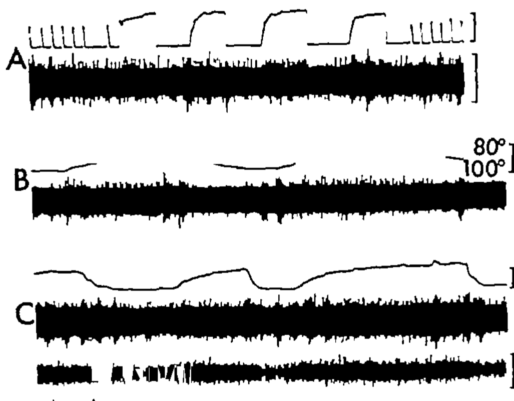


Fig 3 I termitt nt grouped discha ges intermingling w th stretch receptor impulses (positive spikes in nerv bundle supplying the medial gastrocnemius muscle Note la k of i ter pendence between he two types of neural events Same recording sit as in Fig. 1 B and C. A Afferent response t local pressure applied over medial head of the gastrocnemius muscle Stimulus strength indic ted by pward deflection of strai gauge signal (pper trace) B Response t slow passiv movements of the ankle joint. Stret h of calf muscles indicated by upward deflection of signal from ankle goniomet (pper trace) Simultaneous EMG from calf muscles not show in the fig) revealed no activity

C Volu tary ontractions of alf muscles with ankle joint f xed in 90 Positive spike discharge incre ses with lectric l activit in the calf muscles (lower trace) nd with force developed pper t ac whereas spontaneous intermittent bursts occu independently of these ev t Time 2 sec Cal br tion for nerv records 20 µV for EMG 100 µV for strai gauge signal A 9 8 newton C 19 6 newton

In several experiments a correlation was searched for between the intermittent bursts on the one hand and the afferent signals arising in response to peripheral stimuli or voluntary muscle contractions on the other Even though sudden peripheral timuli or volunt ry ontractions occasionally seemed to upset the regularity of the burst sequences, the results were on the whole negati e An experiment of this kind is illustrated in Fig 3 The recording site was the same as in Fig 1 B and C. As judged by the paraesthesiae felt upon needle insertion and the quality and site of the peripheral stimuli required t induce fferent signals, the tip of the electrod was placed in a nerve bundle upplying the medial gastrocnemius muscle Superficial

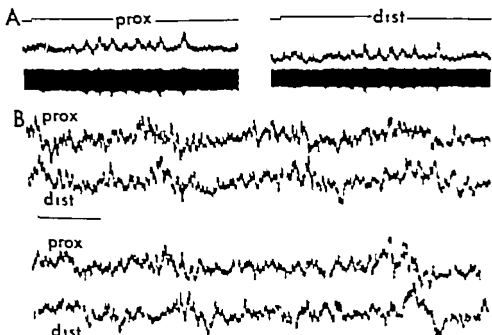


Fig. 4. Grouped discharges in deep branch of right peroneal nerve recorded simultaneously from proximal and distal segment of the nerve. In A the neural events (integrated in the middle trace) are shown on compressed time scale to illustrate approximate synchronism in the periodic appearance of burst sequences in the two segments of the nerve. Signal on upper line serves as common time reference for the proximal and distal records. Parts of the integrated nerve records in A (sections in between dots) are in B shown on expanded time scale in double beam recording. Upper and lower strips in B are continuous run. Note delay of about 100 msec between the proximal and the distal bursts. Time in A 4 sec, in B 0.4 sec. Calibration, 20 µV.

skin stimuli were ineffective but local deep pressure over the medial head of the gastrocnemius muscle caused a marked enhancement of the sustained positive spike activity. However, the intermittent spontaneous bursts continued to appear in their characteristic, periodical fashion, seemingly unaffected by such peripheral stimuli (Fig. 3 A). They were also unaffected by passive movements of the ankle joint and by isometric weak voluntary contractions of the calf muscles (Fig. 3 B and C), manoeuvres which both induced marked changes of the afferent neural discharges. The tests were also negative in the sense that the bursts did not seem to affect the strength of the muscle receptor responses. Thus we [illegible] significant changes in stretch receptor sensibility accompanying the [illegible] of the bursts. Our methods may be objected to in that they d[illegible] very exact estimation of receptor sensibility but still the findings seem [illegible] that neither mechanoreceptor afferents nor fusimotor nerve fibers [illegible] involved in the genesis of the bursts.

By blocking the nerve peripheral to the recording site further evidence was ob-

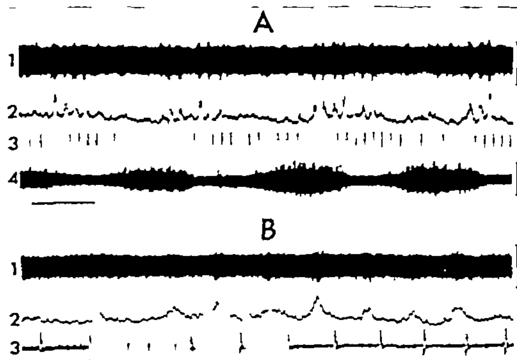


Fig. 5. Pulse and respiratory groupings of discharges in deep branch of peroneal nerve. A. Simultaneous recording of spontaneous neural activity (original in trace 1, integrated in trace 2), ECG (3) and electrical activity of inspiratory muscles (4). Note periodic suppression of bursts during each inspiratory phase. B. Part of the traces in A (sequence in between) shown on expanded time scale to illustrate time relation between QRS complexes and grouped neural discharges. Time in A 5 sec, in B 1 sec. Calibration 20 µV.

tained that the bursts are not derived from impulses in afferent nerve fibres. Bursts recorded from a gastrocnemius nerve bundle in the upper part of the popliteal fossa were not appreciably influenced by a lidocaine block of the tibial nerve in the distal part of the fossa. The block was efficient enough to cause a total paresis of the calf muscles and an anaesthesia of the heel and the sole. Prior to the injection, afferent neural impulses were recorded in response to deep local pressure over the gastrocnemius muscle. In contrast to the bursts, these responses disappeared after the injection.

The experiment illustrated in Fig. 4 provides strong evidence that the grouped discharges are due to synchronized impulses in efferent nerve fibres with slow conduction velocity. The experiment was designed to observe the relation between bursts appearing in two separate recording sites in the same nerve. The two electrodes, leading to separate amplifiers, were inserted 10 cm apart in the peroneal nerve, one at the upper end of the fibula, the other in the middle part of the popliteal fossa. The simultaneous traces in Fig. 4 A show an approximate synchrony both as regards individual bursts and the periodical appearance of burst sequences. However, a closer analysis of the integrated records on expanded time scale shows

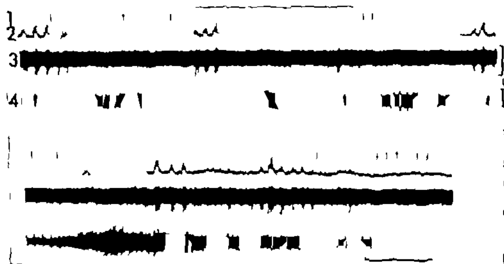


Fig. 6. Pulse and respiratory groupings of discharges in tibial nerve bundle supplying the calf muscles. Upper and lower strips are continuous run. They show from above downwards ECG (1) integrated neurogram ( ), neurogram (3) and EMG from inspiratory muscles 4) After period of regular breathing (upper half) the subject makes deep inspiration and holds his breath for about 15 sec (lower half) See text. Time 5 sec. Calibration for nerve record and EMG 20 µV

that the bursts in the distal recording appear about 100 msec later than those recorded 10 cm more proximally (Fig 4 B) Providing the bursts from the two recording sites derive from impulses in the same set of fibres, their efferent conduction velocity can thus be estimated to about 1 m/sec. This value is low enough to suggest that the bursts originate from impulses in sympathetic rather than in somatic efferent nerve fibres.

Pulse and respiratory rhythms. As originally shown by Adrian, Bronk and Phillips (1932) and Bronk *et al.* (1936) the cardiovascular sympathetic outflow in cats is characterized by a marked grouping of impulses, which often form rhythmic volleys in phase with pulse and/or respiration. The intermittent neural bursts described in the present study showed a similar relationship to pulse and respiratory cycles. Fig 5 A illustrates how sequences of rhythmic grouped discharges in the right deep peroneal nerve (integrated in trace 2) correlate to the ECG signals (trace 3) and the EMG of inspiratory muscles (trace 4) As long as the subject was relaxed and had a regular breathing rhythm, the burst sequences in the peroneal nerve occurred regularly during the expiratory phase and not during inspiration. The burst frequency corresponded to that of the heart beats, even though there were small shifts in the time intervals between each QRS-complex and the succeeding burst (Fig. 5 B) Quite often, one or two bursts dropped out of the sequence and occasionally each second burst dropped out so that the repetition frequency was temporarily halved.

As shown by Green and Howell (1959) the EMG activity as recorded in this way increases in intensity through inspiration and gradually decreases during expiration

In the experiment illustrated in Fig 6 pulse and respiration was recorded simultaneously with neural bursts appearing in a nerve bundle supplying the calf muscles. In this case the bursts were pulse-synchronous also. They appeared in relatively short series at the end of the expiratory phase or the start of the inspiratory phase On other occasions, the series of bursts occurred mainly during the inspiratory phase indicating that even though the burst sequences appear in relation to the respiratory rhythm they are not always coupled to any particular respiratory phase In some experiments, the subject wa instructed to make a deep inspiration and hold his breath for about ?0 sec as illustrated in the lower four traces of Fig 6. Even though no respiratory movements occurred during this period, the respiratory rhythm of the neural discharges seemed to persist. However during the period of breath holding a tonic activity appeared in the neurogram and the burst sequencies were somewhat prolonged The traces also show a slowing of the ECG-rhythm during the initial part of the breath holding period, and it may be noted that there is a parallel change also in the frequency of the neural discharges.

In two experiments, a blood pressure cuff was placed around the thigh and inufflated to about 250 mm Hg The pulse-synchronous bursts in the tibial nerve distal to the cuff persisted d ring the ischemic period which lasted about 1 min. This finding ndicates that the burst are not dependent upon afferent impulses induced by peripher l pulse wa es w thi the extremity

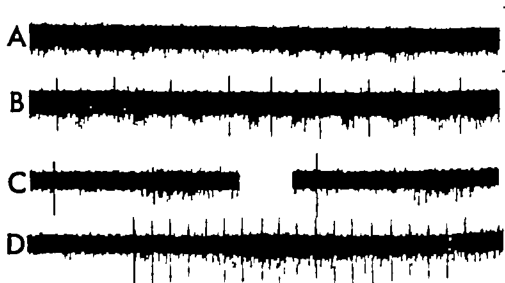


Fig 7 Activ t recorded f om tibial nerv bundle supplying the left gastrocnemius muscle Simultaneous EMG from th m les not show the fig) rev aled no activ t A Control record how ng rela n l ndist nc spontaneous grouping of impulses. B Grouped discharges reflexl d iven l stimula ng lef lnar nerv t ra of 0 5/sec as ndicated by rtefact C Tw of the bu st in B show on expanded me sc le D Sustained neur l activity tibial nerv induced by l a nerv stimul tion t rat of 7/sec Tim A and B 5 sec In C and D 1 sec Cal I on 20 V

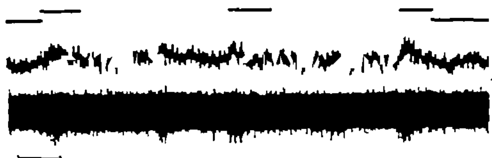


Fig. 8. Neural bursts in tibial nerve branch triggered by Jendrassik manoeuvers, repeated at intervals of about 8 sec (as indicated by signals on upper line). Time 2 sec. Calibration 20 μV

Afferent driving of grouped discharge. It is well known from cat experiments that the pulse and respiratory grouping of the sympathetic outflow is largely due to a driving of the centers by afferent impulses from the sinus and aortic receptors (Bronk *et al.* 1936, Gernandt, Liljestrand and Zotterman 1946, Dontas 1955, Biscoe and Purves 1967). It has also been shown how stimuli applied to a peripheral sensory nerve in the cat may initiate large synchronized volleys of efferent sympathetic impulses (Bronk, Pitts and Larrabee 1939, Sell, Erdelyi and Schaefer 1958). In our experiments we often noticed how a sudden voluntary act or a sudden peripheral stimulus of any kind tended to upset the regularity of the burst sequences. These effects were elusive, however, and often hard to reproduce.

Fig. 7 illustrates how in some experiments neural bursts could be driven by rhythmically repeated, noxious stimuli. Each painful electric stimulus applied to the ulnar nerve triggered a neural burst in the tibial nerve. The bursts appeared with a latency of about 0.7 sec and they could follow frequencies up to about 1/sec. At higher stimulus frequencies, the bursts began to fuse into a more sustained neural activity of low amplitude. Such reflex driving of the bursts by peripheral noxious stimuli was more easily evoked in one subject than in the other.

Some attempts were also made to trigger bursts in lower extremity nerves by sudden clenching of the fists or by sudden Jendrassik manoeuvers. Fig. 8 shows an example of how bursts in the tibial nerve are triggered each time the subject performs sudden Jendrassik's manoeuver. Attempts to repeat these results in a later experiment on the same subject were unsuccessful, however.

Discussion

Two types of arguments support the notion that the spontaneous grouped discharges described in the present paper derive from impulses in efferent sympathetic nerve fibres. First, a series of findings were presented, indicating that the bursts are due to neural events transmitted in efferent nerve fibres which are not skeletomotor and

which (according to one experiment) have a conduction velocity of only about 1 m/sec. Even though further experiments are needed to confirm this value it signifies that the bursts derive from sympathetic rather than fusimotor fibres, a deduction which is also supported by the fact that no significant correlation was observed between the spontaneous bursts and afferent stretch signals from the muscles. Second, the pulse- and respiratory driving of the bursts creates a most characteristic pattern of spontaneous activity previously seen and described by many workers recording from various sympathetic nerves in the cat (for ref see Heymans and Neil 1958, Kahn and Mills 1967 Biscoe and Purves 1967) but never encountered in somatic efferent nerve fibres. To our knowledge, there are no reports concerning spontaneous activity of this type recorded from sympathetic fibres in peripheral muscle nerves of the cat but one might expect that an adequate search for such activity would be rewarding To test our conclusion that the bursts in human muscle nerves derive from sympathetic fibres, it would also be of interest to study the effects of sympathectomy or ganglion blocking agents.

It may seem surprising that our recording method allows the detection of impulses in the small sympathetic fibres. It should be recalled, however that one preganglionic fibre activates many postganglionic fibres (Billingsley and Ranson 1918) and thus each individual deflection in the discharge is probably produced by many post ganglionic fibres functioning as a unit in close synchronism. As originally shown by Bronk *et al* (1936) for the thoracic sympathetic outflow in the cat, there is also a preganglionic grouping of impulses due to a more or less synchronized action of many motor cells in the medullary vasomotor centers, which tend to follow rhythms imposed upon them by afferent nerve volleys from *e.g.* the baroreceptors and distension receptors in the lungs, (*cf.* Biscoe and Purves 1967) Like Bronk *et al* we observed besides the pulse and respiratory rhythms, periods of random or irregular discharges indicating a lability of the central control mechanisms. On the other hand we have not seen regularly recurring waves with a frequency exceeding that of the pulse as reported for the cardiac nerves of the cat. It should be emphasized however that our signal to-noise ratio is so low that grouped discharges of low amplitude may not be detected.

The reflex driving of grouped discharges in the muscle nerves by rhythmic peripheral nerve stimuli also are in accord with the results obtained in studies of the sympathetic outflow in cats Bronk *et al* 1939 Sell *et al* 1958) The long latency of the reflex bursts in the human tibial nerve (0.7 sec) may to a large extent depend upon the long efferent conduction time of the slow sympathetic impulses. It should be noted, however that our estimation of the efferent conduction velocity (1 m/sec) concerned only the pulse-synchronous not the artificially induced bursts. Thus, it is still questionable whether the latter really are of sympathetic origin. This applies also for the sustained neural discharge which appeared during breath-holding and during high frequency stimulation of the ulnar nerve. We cannot exclude the possibility that *e.g.* fusimotor nerve impulses or impulses in muscle spindle afferents contribute to these types of neural responses (see Hagbarth and Vallbo 1968) especially in

view of the evidence that an acti ation or inhibition of the sympathetic system often is accompanied by similar changes in the fusimotor outflow to the muscles (for ref see Gellhorn 1967) On the other hand, since no correlation was found between the appearance of the pulse-synchronous bursts and the sensitivity of the length receptors in the muscle, we have no evidence to support the notions that baroreceptor mechanisms normally operate upon fusimotor outflow (cf Schulte Henatsch and Busch 1959) or that sympathetic outflow normally influences muscle spindle activity (cf and for ref see Eldred Schnitzlein and Buchwald 1960)

The fact that the pulse-synchronous bursts appear preferentially in muscle nerve bundles, seems to agree with the findings concerning differentiated vascular adjustments reflexly induced from the baroreceptors. Löfving (1961 a and b) found that in cats the baroreceptors exert a more intense effect of the vasoconstrictor tonus of muscular vessels than on the constrictor tonus of cutaneous vessels. He ascribes this fact not to regional differences in constrictor fibre distribution but to a regional differentiation of the baroreceptor inhibitory influence upon constrictor fibre discharge. Recent experimental studies have greatly enriched our knowledge about the functional organization of adrenergic and cholinergic vascular control systems (see Uvnäs 1960 a, Folkow 1960) Since baroreceptor reflexes do not operate upon cholinergic vasodilator nerve fibres to skeletal muscles (Uvnäs 1960 b) it does not seem probable that impulses in such fibres contribute to the pulse-synchronous bursts. On the other hand, it cannot be excluded that vasodilator impulses participate in the neural bursts induced by e g noxious stimuli or Jendrassik s manoeuvre (cf Abrahams, Hilton and Zbrożyna 1960 Bolme t al 1967) It would certainly be of interest to see how the events in the electroneurogram correlate to measurements of muscular blood flow g during positional changes of the extremities (cf Roddie and Shepherd 1956)

On the whole the results raise a number of questions which cannot be answered until f rther experiments have been done. It seems, however that this recording method may provide a means for studying the tonus nd reactivity of the sympathetic neural system in alert man, unobscured by the inertia of the effector organs and their reactions to hormonal or local metabolic influences. By analysing the pattern of the sympathetic discharge, continuous information may be gained concerning the degree of synchrony and the rhythms prevailing in large groups of cells in medullary centers, which are known to be invol ed in vital homeostatic functions and which are subject to arious influences from the periphery as well as from higher centers of the brain. It remains to be seen whether it is possibl to recognize characteristic changes in this pattern in patients with diseases in l mg the cardiovascular or central nervous s tem. Considering the relations which exist between certain EEG phenomena and utonomic f nctions (Bonvallet, Dell and Hiebel 1954 Dell, Bonvallet and H geli 1954) t would also be of interest to compare EEG and sympathetic neural patterns under normal as well as pathological conditions

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References

ABRAHAMS, V. C., S. M. HILTON and A. ZBROŻYNA, Active muscle vasodilatation produced by stimulation of the brain stem: its significance in the defence reaction. *J. Physiol.* (Lond.) 1960. *154*. 491—513.

ADRIAN, E. D., D. W. BRONK and G. PHILLIPS, Discharges in mammalian sympathetic nerves. *J. Physiol.* (Lond.) 1932. *74*. 115—133.

BILLINGSLEY, P. R. and S. W. RANSON, On the number of nerve cells in the ganglion cervicale superius and of nerve fibers in the cephalic end of the truncus sympathicus in the cat and on the numerical relations of preganglionic and postganglionic neurones. *J. comp. Neurol.* 1918. *29*. 359—366.

BISCOE, T. J. and M. J. PURVES, Observations on carotid body chemoreceptor activity and cervical sympathetic discharge in the cat. *J. Physiol.* (Lond.) 1967. *190*. 413—424.

BOLME, P., S. H. NGAI, B. UVNÄS and L. R. WALLENBERG, Circulatory and behavioural effects on electrical stimulation of the sympathetic vasodilator areas in the hypothalamus and the mesencephalon in unanaesthetized dogs. *Acta physiol. scand.* 1967. *70*. 334—346.

BONVALLET, M., P. DELL and G. HIEBEL, Tonus sympathique et activité électrique corticale. *Electroenceph. clin. Neurophysiol.* 1954. *6*. 119—144.

BRONK, D. W., L. K. FERGUSON, R. MARGARIA and D. Y. SOLANDT, The activity of the cardiac sympathetic centers. *Amer. J. Physiol.* 1936. *117*. 237—249.

BRONK, D. W., R. F. PITTS and M. G. LARRABEE, Role of hypothalamus in cardiovascular regulation. *A. Res. Nerv. Ment. Dis., Proc.* 1939. *20*. 323—341.

DELL, P., M. BONVALLET and A. HUGELIN, Tonus sympathique, adrénaline et controle réticulaire de la motricité spinale. *Electroenceph. clin. Neurophysiol.* 1954. *6*. 599—618.

DONTAS, A. S., Effect of Protoveratrine, Serotonin and ATP on afferent and splanchnic nerve activity. *Circulat. Res.* 1955. *3*. 363—373.

ELDRED, E., H. N. SCHNITZLEIN and J. BUCHWALD, Response of muscle spindles to stimulation of the sympathetic trunk. *Exp. Neurol.* 1960. 2. 13—25.

FOLKOW, B., Range of control of the cardiovascular system by the central nervous system. *Physiol. Rev.* 1960. *40*. Suppl. 4. 93—99.

GELLHORN, E., Principles of autonomic-somatic integrations. Univ. of Minnesota Press. 1967. 318 pp.

GERNANDT, B., G. LILJESTRAND and Y. ZOTTERMAN, Efferent impulses in the splanchnic nerve. *Acta physiol. scand.* 1946. *11*. 230—247.

GREEN, J. H. and J. B. L. HOWELL, The correlation of intercostal muscle activity with respiratory air flow in conscious human subjects. *J. Physiol.* (Lond.) 1959. *149*. 471—476.

HAGBARTH, K. E. and Å. B. VALLBO, Mechanoreceptor activity recorded percutaneously with semi-microelectrodes in human peripheral nerves. *Acta physiol. scand.* 1967 a. 69. 121—122.

HAGBARTH, K. E. and Å. B. VALLBO, Afferent response to mechanical stimulation of muscle receptors in man. *Acta Soc. Med. upsalien.* 1967 b. 72. 102—104.

HAGBARTH, K. E. and Å. B. VALLBO, Activity in human muscle afferents during muscle stretch and contraction. General discharge characteristics. 1968. To be publ.

HEYMANS, C. and E. NEIL, Reflexogenic areas of the cardiovascular system. J. and A. Churchill Ltd. London 1958. 271 pp.

KAHN, N. and E. MILLS, Centrally evoked sympathetic discharge: a functional study of medullary vasomotor areas. *J. Physiol.* (Lond.) 1967. *191*. 339—352.

LÖFVING, B., Differentiated vascular adjustments reflexly induced by changes in the carotid baro- and chemoreceptor activity and by asphyxia. *Med. exp.* 1961a. *4*. 307—312.

LÖFVING, B., Cardiovascular adjustments induced from the rostral cingulate gyrus with special reference to sympatho-inhibitory mechanisms. *Acta physiol. scand.* 1961b. *53*. Suppl. 184.

RODDIE, I. C. and J. T. SHEPHERD, The reflex nervous control of human skeletal muscle blood vessels. *Clin. Sci.* 1957. *15*. 433—440.

SCHULTE, F. J., H. D. HENATSCH and C. BUSCH, Über den Einfluss der Carotissinus-Sensibilität auf die spinalmotorischen Systeme. *Pflügers Arch. ges. Physiol.* 1959. 269. 248—263.

SELL, R., A. ERDELYI and H. SCHAEFER, Untersuchungen über den Einfluss peripherer Nervenreizung auf die sympathische Aktivität. *Pflügers Arch. ges. Physiol.* 1958. 267. 566—581.

UVNÄS, B., Central representation of vascular control. I. Handbook of physiology. Neurophysiology II. 1960 a. 1131—1162.

UVNÄS, B., Sympathetic vasodilator system and blood flow. *Physiol. Rev.* 1960 b. Suppl. 4. 69—76.

VALLBO, Å. B. and K. E. HAGBARTH, Activity from skin mechanoreceptors recorded percutaneously in awake human subjects. *Exp. Neurol.* 1968. In press.

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The Effect of Pulmonary Artery Occlusion on the Sulfomucopolysaccharide Synthesis and Collagen Content in Rat Lungs

By

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Abstract

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I rats the left branch of the pulmonary artery was occluded. The incorporation of ^{35}S in lungs was studied till the eleventh day after operation. The hexosamine and hydroxyproline content of the lungs was determined on the first and fourth day after occlusion. The incorporation of ^{35}S is significantly increased on the fourth day and is maximal on the sixth day. Eleven days after operation the incorporation is significantly lower than on the sixth day. During the time studied the hexosamine and hydroxyproline content of the occluded lungs increased, but the difference was significantly changed only for hexosamine after the fourth day. The significance of the results obtained for the understanding of arious pathologic changes is briefly discussed.

General and localized pulmonary fibrosis is a well known sequel of many forms of pulmonary disease such as pneumonia, pulmonary infarction, tuberculosis, sar coidosis and pneumoconiosis. It is also a common complication to X-ray treatment of the thoracic region. Although the development of fibrosis seems to be a charac teristic pulmonary response in various pathological conditions, the biochemical mechanisms nvolved in pulmonary fibrosis are poorly nderstood. The formation of collagen fibrils i normal tissue as well as in vario types f scar tissues and granuloma tissues seems to be closely related to the biosynthesis of acid muco-polysaccharides (J nge Hülsing 1959 Matthews 1965) The composition and distribution of these compound in the lungs ha e earlier been studied to some ex tent with chemical methods (Lieflander *et al* 1963 Perić Božović *et al* 1965 and Pierce 1964) W th regard to the rate of biosynthesis of these compounds in the

lungs under normal and pathological conditions, interesting observations with experimental silicosis have been reported by Hauss et al. 1963

In the present investigation the effect of impairment of the functional pulmonary circulation on the biosynthesis of sulfomucopolysaccharides in normal lung tissues of the rat was studied. The functional circulation to the left lung was stopped by ligation of the left pulmonary artery. For the elucidation of sulfomucopolysaccharide synthesis the technique with measuring of ^{35}S-sulfate incorporation in these compounds after injection of the isotope as inorganic sulfate was used.

Materials and methods

I the experiment thirty female albino rats, 150—200 g b.w. were used. The left branch of the pulmonary artery of all animals was ligated under ether anesthesia. After skin incision the thoracal cavity was opened by cutting the muscles of the fifth intercostal space and the left branch of the pulmonary artery was feed from the bronchi, the artery was ligated and the thoracic cavity losed as quickly as possible with one suture. Few animals had to be alsted with artificial respiration in order to recover to spontaneous respiration after the operation. After recovery from the anesthesia, the animals did not show ny ppreciable respiratory difficulties.

The operated animals were divided int four groups. Each animal was given 0.5 mC of S^{35} per 100 g b.w. intraperitoneally in the form of $Na_2S^{35}O$ and in olume of 0.4—0.5 ml. The first group of animals was injected 1/2 hr af er operation, the second 3 days, the third 5 days and the fourth 10 days af er occlusion of the artery. Lung tissue was taken from the right control lung and the left occluded side 4 hrs after the injection. Care was taken not to include the tissue in the vicinity of the hilus in the samples.

Samples of lun tissue were left in acetone during 48 hrs and then dried in acuum for 4 hrs. Dried tissue was homogenized in mortar and samples of 10 mg were weighed. To each sample was added mg of bacterial Pronase in 1 ml of tris-buffer pH 6.9—7.0) and the sample was digested for 1 hrs at 45 C. The solution was centrifuged and 100 µl was pipetted on aluminum plates. The pla es were kept at 100 C for 1 h and dried. The radioactivity was measured with micro end-window Geiger Müller counter (Boström et l. 1964).

I the first two groups of animals the hydroxyproline nd hexosamine content was determined in the tissue samples. Hydroxyproline was determined by the method of Neuman and Logan 1950 as modified by Bergman and Loxley 1963. L-hydroxyproline (British Drug Houses LTD and purified collagen Sigma Chemical Co as standards were subjected t the same procedure as he tissue samples. Hexosamine was determined by the Elson and Morgan colorimetric method as modified by Boas 1953 and Lindstedt et l 1965. As tandard D-g ucosamine used.

For tatistic alculation the student T-test was used.

Results

The results of the measurements of S^{35} ncorporation are presented in Table I.

In animals injected on th da of peration the incorporation of S^{35} is 19 per cent higher in the lung with the occluded pulmonary artery as compared to the control side, but the differenc is not statistically ignificant. The fourth and sixth day after operation the incorporation of S^{35} is 46 per cent and 65 per cent respectively higher in the lung with ccluded pulmonary artery and these increases are significant. On the eleventh da the incorporation is still higher (23 per cent) in the "occluded" lung but the difference is no longer significant. If the incorporation of S^{35} is considered in the l ngs with occluded artery and compared on different da s, then the increase *from the first t the fourth day is gnifica t ($p<0.01$)* but the further increase from the fourth t the sixth da is no longer ignificant. The de-

TABLE I. Effect of the pulmonary artery occlusion on the incorporation of S^{35} in the lungs of rats.

Number of rats	Time after ligation of the artery	Mean CPM/mg of dry tissue ± S.E.		p
		Right side (control)	Left side (occluded)	
6	1 days	510 ± 23	600 ± 35	>0.05
6	4 days	580 ± 5?	850 ± 73	<0.01
10	6 days	600 ± 33	990 ± 109	<0.01
8	11 days	560 ± 60	690 ± 67	>0.1

Standard error of the mean

crease in the incorporation from the sixth to the eleventh day is significant ($p < 0.05$)

The results of the hydroxyproline and hexosamine determinations are shown in Table II

There is only a significant difference in the hexosamine content in animals on the fourth day after the operation.

Discussion

The rate of ^{35}S-sulfate incorporation in the sulfomucopolysaccharides of mesenchymal tissues has been considered to reflect the biosynthesis of these compounds (Boström 1960) It is known to be influenced by many factors such as age (Junge-Hülsing 1965) administration of hormones (Asboe Hansen 1963) and drugs (Boström 1966) presence of various specific and unspecific mesenchymal reactions etc., as indicated by numerous studies on cartilage, skin, vessel walls, and heart valves of animal and human origin (Boström *et al.* 1963 Junge-Hülsing 1965) Also in certain parenchymatous organs, *e.g.* liver (Junge-Hülsing and Hauss 1960) and

TABLE II. Effect of the pulmonary artery occlusion on the hexosamine and hydroxyproline content in dry tissue of rat lungs.

Number of rats	Time after ligation of the artery	Per cent Hexosamine in dry tissue		p	Per cent Hydroxyproline in dry tissue[1]		p
		Control	Occluded		Control	Occluded	
6	1 days	0.88 ± 0.158	1.03 ± 0.103	>0.05	0.70 ± 0.062	0.61 ± 0.053	>0.05
6	4 days	0.76 ± 0.038	0.98 ± 0.067	<0.02	0.69 ± 0.081	0.61 ± 0.068	>0.05

1 mg of collagen standard gave 60 μg hydroxyproline after the same procedure.

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The Mechanisms behind the Rapid Blood Volume Restoration after Hemorrhage in Birds

By

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Abstract

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The mechanisms responsible for the more efficient volume restoration after hemorrhage in birds as compared with mammals were studied in experiments on ducks. The results suggest that *qualitatively* similar mechanisms operate to mobilize tissue fluid in ducks as in cats (see Öberg 1964). The most important factor seems to be a strong reflex vasoconstriction in the skeletal muscles, increasing considerably the pre- to postcapillary resistance ratio with a fall in capillary pressure as a result. In this way net fluid absorption is reflexly initiated in the tissue which contains the largest tissue fluid reservoir in the body. At the same time the precapillary sphincters become secondarily relaxed, increasing the capillary surface area available for absorption. When in the course of a large blood loss arterial and venous pressures become reduced as well, this will further lower capillary pressure and do so in all tissues, thus enhancing fluid absorption. The results indicate that, from a *quantitative* point of view, considerable differences exist between ducks and cats. First, the reflex vasoconstriction and the subsequent capillary pressure fall are more pronounced in duck skeletal muscle. Second, the capillary surface area is approximately three times larger in duck muscle. These circumstances imply especially favourable conditions for rapid fluid absorption. It is probable that the greater resistance to blood loss and shock of most bird species as compared with mammals is directly related to their much greater capacity for fluid mobilization from the skeletal muscles.

It has previously been shown that most birds, especially pigeons and ducks, tolerate hemorrhage much better than mammals though pheasants appear to be an exception in this respect (Kovách and Szász Mikolás 1967, Kovách, Szász Mikolás and Pilmayer 1967). Thus, hemodilution after bleeding often occurs more rapidly and to a greater extent in the pigeon than in the rat (Kovách and Bálint 1967).

In mammals the dilution of the blood after bleeding is the result of an absorption of tissue fluid across the capillaries subsequent to a fall in capillary pressure. It is obvious that any fall in central arterial and/or venous pressures will tend to lower

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capillary pressure as well, thus initiating fluid absorption. It has, however recently been shown that such a fall in capillary pressure can be reflexly induced also by means of a neurogenic increase in the ratio between the pre- and postcapillary resistances. This specific reflex adjustment of the pre- to postcapillary resistance ratio occurs predominantly in skeletal muscle, here producing a reduction of capillary pressure with a consequent fluid absorption, without necessarily involving any appreciable reduction of central arterial and venous pressures (Öberg 1963, 1964). It therefore has the advantage to initiate fluid absorption at such an early stage during a blood loss that the overall reflex adjustments of the cardiovascular system are still capable of maintaining central arterial and venous pressures largely unchanged. The question arises whether such a mechanism can also account for the often very rapid restoration of the blood volume seen in birds, or whether additional mechanisms have to be considered.

Folkow Fuxe and Sonnenschein (1966) found that muscle blood flow and the capillary surface area available for fluid exchange, measured as the capillary coefficient (CFC) are decidedly higher in the duck than in the cat. They f rther found that reflex increases in muscle blood flow resistance can be extremely powerful in the duck. The possibilities for drastic reflex reductions of the capillary pressure and net fluid absorption in this tissue therefore seem to be at hand. Furthermore, since skeletal muscles makes out a considerable fraction of the body mass also in ducks, it seems *a priori* likely that a major part of the interstitial fluid, which dilutes the blood after hemorrhage, comes from this tissue in the duck also. In order to explore whether the mentioned type of reflex vascular adjustments really are powerful enough to account for the rapid restoration of the blood volume in the duck after hemorrhage, a series of experiments were ndertaken where reflex vascular adjustments and the rate of net fluid transfer across the capillaries were studied in th duck skeletal muscle after graded bleedings.

Methods

Experiments were performed on altogether 11 ducks, with body weights ranging between 2.1—3.5 kg.

I 8 ducks changes in muscle blood flow CFC and tissue olume were continuously recorded after graded bleeding. Tw of them were decerebrated, while the other [illegible] were nesthetized either with chloralose 40—60 mg/kg, dissolved in 5 % borax solution, or with pentobarbital sodium, 30 mg/kg b.w. Additional amounts of pentobarbital were given [illegible] as necessary.

The trachea was cannulated, the right jugular ein and the left brachial artery were exposed for subsequent cannulation. The right femoral artery and ein with the nerv supply to the calf muscles were isolated, care being taken not to damage the sciatic nerv or the nerv filaments round the artery. The thigh muscles were tied off and cut owards the lower end of the femur and the leg was autiously prepared free from the skin dow [illegible] the ankle where tight ligature was placed so as to eliminate the foot from the circula ion. The femur was then divided just above the knee joint and its marrow cavity pl gged w th bone wax. The isolated calf muscles were enclosed [illegible] plethysmograph, using the [illegible] vert ght seal. The preparation losely resembles that of Kjellmer (1965 for [illegible] muscles.

After heparinization the venous effluent from the alf m [illegible] le [illegible] measured with silicone filled photo-electric drop chamber and recorded on [illegible] k [illegible] graph by means of an ordinat writer. The height of the tube, draining the drop hambe [illegible] d be adj sted so as to set the venous outflow pressure [illegible] any wished level. I this w [illegible] he [illegible] f p paration could be set an "isovolumetric le [illegible] t he start of the experiment, alt [illegible] ev luations of eflex decreases

in mean pillary pressur nd also intermittent determinations of the capillary filtration coefficient (CFC) (for details see Kjellmer 1965, Folkow Fuxe and Sonnenschein 1966) The venous effluent was then collected in f nnel and returned to the animal ia the jugula ein Arterial blood pressure was continuously recorded with a mercury manometer connected to a po ethylene catheter inserted in the left brachial art ry I the course of the experiment the vasomotor fibres to the calf essels could be blocked by the application of xylocain around the essels and the sciatic nerv Bleeding was performed through a cannula in one of the carotid arteries. The hematocrit (Hct) wa measured before and after the bleeding t regular intervals to follow the rate and extent of the dilution of the blood.

I three anesthetized ducks arterial blood pressure heart rat cardiac output (C.O) thermodilution method) and hematocrit were measured before d ring and after the loss of p to 25 pe cent of the calculated blood olume In order t explore the rate t which the restoration of the blood olume could tak place During a brief period of nesthesia the animals were prepared as earlier described by Folkow Nilsson and Yonce (1967) The tissues were infiltrated with xylocain t the sites where cann les had been inserted.

Results

I Anesthetized ducks Typical hemodynamic changes in the calf muscles after a blood loss are illustrated in Fig 1 Panel A shows the situation before hemorrhage Hct was 41 per cent and resting calf blood flow was about 30 ml/min × 100 g at a mean arterial pressure of 90 mm Hg CFC was 0.05 ml/min × 100 g × mm Hg and the venous outflow pressure had been initially adjusted so as to create an isovolumetric state in the calf muscles.

After a rapid withdrawal of 35 ml of blood, about 11—12 per cent of the predicted blood volume (being around 10 per cent of the body weight in ducks Andersen 1966 the blood pressure fell initially to about 70 mm Hg to rise to 80 mm Hg ithin a few minutes, with a further gradual rise towards control during the subsequent 10 min. Calf blood flow fell to about 7 ml/min 100 g implying a more than threefold increase in regional flow resistance At the same time CFC increased to 0 13 lose to the maximal level for duck muscle (Folkow Fuxe and Sonnenschein 1966 The calf olume showed a rapid, tead decline indicating a considerable absorption of tissue fluid of th order of 0.5 ml/min 100 g of muscle (panel B of Fig 1 taken few minutes after the blood loss compare the difference in the slope of the volume recordings in panels A and B) Since CFC as well as the initial rat of fluid absorption was known, it can be calculated that mean capillary pressure had fallen about 4 mm Hg in panel B as compared with panel A Hct was at this earl posthemorrhagic tage 40 per ent nd 90 min later 37 per cent imply ng a fluid addition after this period of about 70 per cent of the blood loss.

The reflex nature of the mentioned hanges was revealed by the effect of vasomotor nerve blockade in the alf preparation (panels C nd D of Fig 1) The shed blood had here been retransf sed a new teady tat established nd the sciatic nerve and the ascular pedicle t the calf had been infiltrated with xylocain Panel C shows the new control situation bout ne hour after A and B, w th an isovolumetric state a blood pressure of 90 mm Hg muscle blood flow of 25 ml/min × 100 g and a CFC of 0.09 Thus despit the nerv blockade flow is now slightly lower than in A indicating that sympat tic t ne w probably ery low in A. The lower

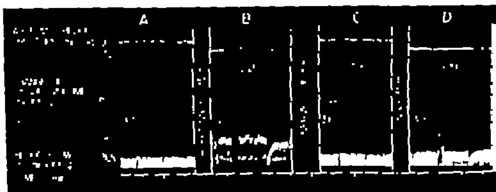


Fig. 1. Duck 3 0 kg; pentobarbital anesthesia. Effects of graded bleeding on blood flow re sistance capillary filtration coefficient (CFC) and transcapillary fluid exchange in the calf muscles before (panels A and C) nd immediately after (panels B and D) blood loss of 35 ml. Panels A and B illustrat the situation when the vasomotor nerves t the calf muscles are intact; panels C and D when these nerves are blocked. The change in slope of the olume recording, here indicated by the angle between the drawn lines, illustrates the rate of regional fluid absorption after blood loss in the tw situations. Note the marked reduction with respect to both resistance increase and rate of fluid absorption from the calf muscles when the regional vasoconstrictor fibres are blocked. For details, see text.

flow in C may reflect a reduced metabolism of the calf muscles after the nerve blockad resulting in a slightly higher "basal vascular tone"

Panel D shows the situation a few minutes after withdrawal of 35 ml of blood. In this early posthemorrhagic period blood pressure had fallen to about the same extent as when the animal was bled before the vasoconstrictor fibre block (panel B) but the flow resistance in the calf had now only increased ten per cent, at most. This implies that the pre to postcapillary resistance ratio could hardly ha e changed significantly This was also clear from the f ct that the rate of fluid absorption from the calf was now only some 25 per cent of that obtained with hemorrhage before the nerve blockade as shown in panel B. The slight rise in flow resistance may have been due to increased amounts of blood-borne catecholamines Since the flow reduc tion was so small the secondary relaxation of the precapillary sphincters could be expected to be f less pronounced than in the situatio before the nerve block, as indicated by the fact that CFC did not change significa tly between panels C and D These results will be f rther discussed below — Essentially similar results were obtained in all the ducks.

2. *Unanesthetiz d du ks* Since unanesthetized ducks usuall maintain their ar terial blood pressure after bleeding better tha nesthetized ones, t seemed likely that the reflex vasoconstriction in keletal muscl w th consequent lowering of capillary pressure b more powerful in the otherwise nta t organism, if anything An even more rapid autotransfusion of tissue fl id f om he sk letal muscles after a blood loss should thus be expected n the wak nimals

Three control experiments were performed n naesth tized animals. Up to 25 per cent of th blood vol me could here be emo ed w th ut any marked fall n

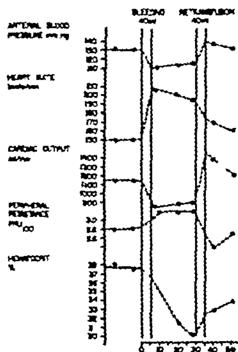


Fig. 2. Mean values of changes in arterial blood pressure, heart rate, cardiac output, total peripheral resistance and hematocrit in 3 unanesthetized ducks when exposed to a rapid blood loss of 40 ml with retransfusion 25 min later. Note especially the very rapid decrease of hematocrit after the blood loss. For details, see text.

arterial pressure. Fig. illustrates the mean values from the three ducks, exposed to a rapid blood loss of 40 ml of blood (13—15 per cent of the predicted blood volume) which was retransfused after 25 min. After this blood loss mean arterial pressure fell about 15 per cent while heart rate increased from 150/min to about 200/min. C.O. fell on average to about 75 per cent of control, implying an increase of total peripheral resistance of only some 15 per cent, which will be further discussed below.

The steep decrease in Hct during the 25 min posthemorrhagic period (see Fig. 2 from 3 —38 to 30) implies a rapid absorption of tissue fluid. The addition of fluid to the blood corresponds to some 50 ml, when calculated from the difference in Hct prior to the blood loss and that present 20—25 min after blood loss. This apparent "overcompensation" is presumably explained simply by slight errors in the Hct determinations. When the blood loss is instead computed from control Hct and the Hct value obtained immediately after retransfusion of the shed 40 ml of blood, one arrives at an addition of fluid of some 30 ml. Even if both these figures are probably somewhat incorrect, they together suggest that there is a largely complete restoration of the blood volume by hemodilution already 20—25 min after a 13—15 per cent blood loss in the awake ducks.

Discussion

Earlier studies (Kovach and Szasz Mikolas 1967) have shown a much better tolerance to hemorrhage in the pigeon as compared with the rat, with a more extensive transfer of tissue fluid into the intravascular compartment after blood loss in the

former species. The present study was undertaken to elucidate in which tissues of the body this rapid fluid mobilization mainly occurs and what is the explanation of the obvious difference of the efficiency of the involved homeostatic mechanisms.

Folkow, Fuxe and Sonnenschein (1966) demonstrated that CFC of the skeletal muscles in ducks is much higher than in the cat. Reflex cardiovascular adjustments producing identical changes in capillary pressure would therefore result in a correspondingly more rapid fluid transfer in the duck. Thus, it appeared *a priori* likely that the absorbed fluid originated mainly from the skeletal muscles, where it is known from experiments on cats that reflex constrictions normally involve an increase of the pre- to postcapillary resistance ratio that lowers capillary pressure (Öberg 1963, 1964).

The recordings from the experiment illustrated in Fig. 1 reveal that the muscle resistance vessels of the duck were reflexly increased to a considerable extent after blood loss, producing a threefold increase in regional resistance. It was combined with a marked CFC increase (from 0.05 to 0.15), which was presumably due to a secondary relaxation of the precapillary sphincters (*e.g.* Cobbold *et al.* 1963, Öberg 1964). It could furthermore be calculated that the capillary pressure fall, of the order of about 4 mm Hg, had been caused predominantly by a reflex increase in the pre- to postcapillary resistance ratio, amounting to some 35 per cent.

This was also illustrated by the fact that after blockade of the vasoconstrictor fibres the fall in mean capillary pressure, for the same blood loss and reduction in arterial pressure, could be calculated to be only about 25—30 per cent of that seen while the vasoconstrictor fibre control was still intact. Therefore, in this situation also some absorption of tissue fluid occurred, though at a much slower rate. This is in agreement with the fact that, other things being equal, any lowering in central arterial and venous pressures will lower capillary pressure as well and thus *per se* favour fluid absorption. Such factors may be expected to become increasingly important after large blood losses, simply because the reflex mechanisms can then no longer hinder a drastic fall in arterial and central venous pressures. On the other hand, an arterial pressure fall alone is usually less efficient in this respect since precapillary autoregulation tends to minimize the fall in capillary pressure if no reflex adjustment of the resistance vessels is superimposed (Öberg 1964). Because of the techniques used in the present experiments venous outflow pressure in the calf preparation was kept constant. However, it can be expected to fall in the intact animal whenever the blood loss is substantial enough to markedly lower central venous pressure, and this factor inevitably adds to the reduction in mean capillary pressure.

In case the initially rapid absorption of fluid, shown in Fig. 1, had been maintained unchanged with time and involved the entire skeletal muscle mass, it would have meant a complete replacement of the shed blood with tissue fluid within ten minutes. However, subsequent sections of the recordings revealed that the rate of absorption soon began to decline. There were further clear signs of a gradual reduction of the reflex response along with the regain of blood volume, but still 20 min

after the b eeding muscle flow resistance remained about doubled as compared with the control situation. However the absorption of fluid had at this time largely subsided, the volume curve being again nearly isovolumetric despite the increased flow resistance. This is presumabl a consequence of the by now reduced colloid osmotic pressure of the blood and the increased tissue colloid osmotic pressure, both being factors that tend to coun'eract the effect of the lowered capillary pressure. To judge from the Hct alues about 70 per cent of the shed blood had nevertheless been re placed by means of tissue fluid absorption after about 20 min in the anesthetized duck provided that no erythrocytes had simultaneously been mobilized to the cir culating blood, in which case the absorbed fluid volume must have been even larger)

In the awake ducks the absorption of tissue fluid was, if anything even more efficient to judge from the Hct changes. At the same time the increase in *t tal* flow resistance was surprisingly small, only about 15 per cent, after the moderate blood loss, which calls for some comments. A discussed above flow resistance increased markedly in the calf muscles of the anesthetized duck upon moderate blood loss. To judge from experiments on cats the reflex increase in flow resistance upon graded unloading of the baroreceptors is usually most pronounced in the skeletal muscles (Löfving 1961) This seems to be the case als after bleeding at least in the early posthemorrhagic situation and when the blood loss is not so extensive as to cause a marked fall in blood pressure Öberg 1963 1964) It is therefore possible that the relatively small increase in total flow resistance, seen in the awake ducks after moderate bleeding conceals a substantial and relatively selective increase in muscle flow resistance Again, to draw a parallel to the cat the increases in regional resistance e g the kidneys, the gastrointestinal tract or the skin is usually decidedly smaller upon unloading of the baroreceptors, at least as long as no additional stimuli excite the vasomotor centre Löfving 1961 Follow Johansson and Löfving 1961) On the other hand the flow resistance of e g the coronary Mitáčová, Kovách and Bird 1965 and cerebral circuits *decrease* somewhat whenever blood loss occurs.

During resting steady state total muscle blood flow may be predicted to amount to some 300 ml in the duck, if flow/min 100 g of muscle is about 30 ml (see Fig 1 Suppose that the muscle vascular circuit is reflexly constricted so as to reduce its blood supply t about one third while the sum of the resistances of the other systemic circuits changes only little, as seems to be the case in the cat upon partial unloading of the baroreceptors or after a moderate blood loss. Such a pattern of reflex change would decrease C.O only some 15—20 per cent from the average resting control alue which, in fact, coincides well with th actual figures given in Fig 2 for the awake duck.

It is obvious that a reflex increase of the pre to postcapillary resistance ratio (Öberg 1963, 1964 implies a most efficient way of causing a rapid blood volume restoration, evidently operating in the duck also to judge from the present results. This mechanism has the advantage that it is capable of exerting its influence even while the central arterial and venous pressures ha e not et been ppreciably reduced by the blood loss and therefore it is probably of considerable importance in the

normal homeostasis of the blood volume. When, in addition, arterial and venous pressures fall in the course of a large blood loss this will obviously add to the fall in capillary pressure and therefore further enhance the fluid absorption.

Thus, the present results suggest that the same mechanisms of compensation for hemorrhage are present in ducks as in cats, though this compensation is more rapid and efficient in the bird species mainly for two reasons. First, the reflex lowering of the mean capillary pressure level, adjusting the Starling equilibrium towards net fluid absorption, seems to be even more effective in the duck, if anything, to judge from the extensive increases in muscle flow resistance that can occur in this species (Folkow, Fuxe and Sonnenschein 1966). Second, the capillary surface area that is available for this absorption, which mainly seems to occur in the skeletal muscles, is approximately three times larger in the duck than in the cat. This large capillary surface area will, of course, be of advantage also when a blood loss becomes so extensive as to produce considerable reductions in central arterial and venous pressures as well, with a consequent further enhancement of the capillary pressure fall.

These results and considerations appear to fully explain the, at first sight, surprising ability of some bird species to rapidly restore their circulating blood volume by absorption of interstitial fluid. The present results agree with others, obtained by measuring the hemoglobin content and protein level in the blood after bleeding (Kovách and Bálint 1967). It is probable that the resistance to blood loss and the fluid transfer capacity are directly related to each other. The fact that many birds less easily are brought to the level of irreversible shock than most mammals may to a great extent be the result of their better capacity to compensate for reductions of the blood volume. However, not all birds react equally efficiently in this respect, the most efficient homeostasis being achieved in flying and diving birds, such as the pigeon and the duck.

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References

ANDERSEN, H. T.: Physiological adaptations in diving vertebrates. *Physiol. Rev.* 1966. *46* 212—243.

COBBOLD, A., B. FOLKOW, I. KJELLMER and S. MELLANDER: Nervous and local chemical control of precapillary sphincters in skeletal muscle as measured by changes in filtration coefficient. *Acta physiol. scand.* 1963. *57* 180—192.

FOLKOW, B., K. FUXE and R. R. SONNENSCHEIN: Responses of skeletal musculature and its vasculature during "diving" in the duck. Peculiarities of the adrenergic vasoconstrictor innervation. *Acta physiol. scand.* 1966. *67* 327—342.

FOLKOW, B., B. JOHANSSON and B. LÖFVING: Aspects of functional differentiation of the sympatho-adrenergic control of the cardiovascular system. *Med. exp.* 1961. *4* 321—328.

FOLKOW, B., N. J. NILSSON and L. R. YONCE: Effect of "diving" on cardiac output in ducks. *Acta physiol. scand.* 1967. *70* 347—361.

KJELLMER, I.: On the competition between metabolic vasodilatation and neurogenic vasoconstriction in skeletal muscle. *Acta physiol. scand.* 1965. *63* 450—459.

KOVÁCH, A. G. B. and T. BÁLINT: Hemodilution after hemorrhage in pigeon and rat. *Acta physiol. Acad. Sci. hung.* 1968. In press.

Kovách, A. G. B., A. Mitsányi and W. Stekiel, Local blood flow and oxygen tension of brain tissue in hemorrhagic shock. *Acta physiol. Acad. Sci. hung.* 1965, 25, 35.
Kovách, A. G. B. and E. Szász-Mikolás, Survival of pigeon after graded hemorrhage. *Acta physiol. Acad. Sci. hung.* 1965. In press.
Kovách, A. G. B., E. Szász-Mikolás and N. Pilmayer, Comparison of different birds and mammals in respect of resistance to hemorrhage. *Acta physiol. Acad. Sci. hung.* 1965. In press.
Löfving, B., Cardiovascular adjustments induced from the rostral cingulate gyrus. *Acta physiol. scand.* 1961, 53, Suppl. 184.
Mitsányi, A., A. G. B. Kovách and Z. S. Biró, Myocardial blood flow in haemorrhagic shock with and without phenoxybenzamine treatment as determined by the "heated thermocouple" method. *Acta physiol. Acad. Sci. hung.* 1965, 25, 36.
Öberg, B., Aspects on the reflex control of capillary filtration transfer between blood and interstitial fluid. *Med. exp.* 1963, 9, 49—61.
Öberg, B., Effects of cardiovascular reflexes on net capillary fluid transfer. *Acta physiol. scand.* 1964, 62, Suppl. 229.

Acta physiol. scand. 1968. 74. 123—133

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Effect of Noradrenaline on the Elimination of Exogenous Lipids from the Blood

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Abstract

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The rate of elimination from the blood stream of a fat emulsion, used for parenteral nutrition, was studied in 12 dogs before and during noradrenaline infusion at constant rate. The volume of distribution of the injected emulsion (the lipid space) was larger than and highly correlated to the plasma volume. The noradrenaline infusion reduced both the plasma volume and the lipid space in all the dogs, excluding two in which an increase was observed. The zero order rate constant (K_1 mmoles/l plasma/min) for the elimination of the emulsion-triglycerides at high concentrations varied inversely with the change in plasma volume (= 0.93). K × plasma volume was not influenced by the noradrenaline infusion. The first order rate constant (K_2 per min) for the elimination of emulsion-triglycerides at low concentrations decreased during the noradrenaline infusion in all the dogs, except two which had increased plasma volume. In these two dogs K increased. In 5 dogs the relation between K and plasma volume was studied before and after a plasma transfusion. The results indicate that the product K × plasma volume was constant. The nature of the rate constants K and K_2 are discussed in terms of metabolic physiology, the relation between enzymatic activities, the rate of transportation of substrate, and the arations in volume of distribution of the substrate.

The kinetics *of elimination from the blood stream of chylomicrons and an artificial* fat emulsion has been described by two rate constants and one concentration, C (Carlson and Hallberg 1963, Hallberg 1965) One rate constant, K_1 is an expression for the maximal removal rate (mmoles/l plasma/min) apparent at high plasma triglyceride concentrations. The other rate constant, K describes the fractional removal rate (per min) present at low concentrations. The concentration C (mmoles/l) is the concentration where the amount of lipid eliminated by first order kinetics $(K \times C)$ equals K $(K = K \times C)$.

A hypothesis was presented in which it was suggested that K was an expression for the amount of available enzyme responsible for the process of elimination (lipoprotein lipase). This enzyme should be saturated at the high blood concentration.

Furthermore it was suggested that K was an expression for that fraction of the volume of distribution of the substrate (the exogenous lipids) which per unit of time was cleared from the s bstrate (Hallberg 1965 a) It is therefore of interest to consider the hypothesis in the light of factors influencing the rate constants.

There are several studies which link the elimination of chylomicrons from the blood stream to the occurrence of the enzyme lipoprotein lipase (*cf* Dole and Hamlin 1962, *cf* Rodbell and Scow 1965 Hallberg 1967)

In tissues there are also other lipases which hydrolyze tissue triglycerides, *g* in the adipose tissue Noradrenaline is one factor that is known to enhance the lipolysis in adipose tissue (*cf* Havel 1965) The interdependence of different lipases is less known. It was therefore of interest to study the effect of noradrenaline on the rate constant K_2 for the exogenous triglycerides. It is also known that catecholamines cause a change in the blood flow through tissues (Goodman and Gilman 1958) Therefore, noradrenaline might influence the fractional removal rate constant K

It is of interest also from a clinical viewpoint to study the elimination from the blood stream of fat em lsions used for parenteral nutrition in a traumatic stat umulated by means of noradrenaline

Material and methods

Mongrel dogs of both sexes were studied in the morning after fasti g overnight. Anesthesia was induced with Nembutal® (30 mg/kg Abbott) and the dogs were tracheally int bated. The anesthesia was continued wit small mounts of Nembutal, repeated when necessary The rat of elimination of exogenous lipids from the blood stream was studied twice before nd during constant rat infusion of noradrenaline (Noreadrin Conc ® Astra, Sodertälje Sweden) diluted with saline

The exogenous lipid was f t emulsion clinically used for parenteral nutrition (I tralipid® 20 % V trum, Stockholm) The jected amou ts of lipids (bout 0 3 g TG/kg b. w) and noradrenaline are given in T ble I

The fat emulsion and the noradrenaline were given through tw catheters inserted through small peripheral eins far into central ein. Blood was sampled from third catheter in branch of the femoral artery

The noradrenaline infusion was started, when the first injected dose of lipids had leared from plasma, as determined isually from the opalescence of plasma. After 20—30 ml the second lipid jection was given, and the first blood sample was taken 10 min later (30- 40 mi after the start of the noradrenaline nfusion)

The plasma olume was determined twice in ll the dogs, mmediately before each injec tion of the fat emulsion by injection of huma [131] I albumin (kindly supplied by L. O Plantl t King Gustaf V Research Institut I few cases third determination of the plasma ol me was made t the end of the noradrenaline infusion (Table I)

A separat tudy of the elimination of the fat emulsion in 5 anesthetized dogs, fasted ov night, was performed before and after transfusion of plasma. Fresh plasma was obtained from anesthetized dogs, fasted overnight, on the day of the experiment by sampling arterial blood into siliconized blood bottles w th ACD solution (4/1 A containing citric acid, 0.48 g trisodium citrate 1 32 g and glucose 1 47 g per 100 ml of ACD-sol tion) After centrif ga-tion, the plasma wa immediately transferred empty bottles and then transfused The plasma infusions were given for abou 30 min and he ecipients were then allowed to rest for 60 min t avoid effects of echolamine present in the transfused plasma. The plasma olume was determined before each injection of the fa emulsion.

Analys s

Arterial blood was collected into heparinized tubes and centrifuged (Hallberg 1964) Plasma was extracted immediately after centri gation for determination of free fatty acids (FFA) (Trout *et al.* 1960)

The amount of exogenous triglycerides i plasma was determined from the top fraction after separation of the plasma triglycerides in duplicates i polyvinyl-pyrrolidone (PVP) density gradients (Gordis 1962, Hallberg 1964)

Triglycerides were determined in triplicates by Carlson (1963) method.

The plasma olume was determined with human ¹³¹I-albumin (Wetterfors 1965) by taking blood and plasma samples befor and 9 min after the ¹³¹I-albumin injection.

I few cases the lipoprotein lipase activity (LLA) in plasma wa determined before and during the noradrenaline infusion by Boberg and Carlson (1964) method.

Definitions

1 *The amou t of tot l available activity (lipoprotei lipase acti ity?)* responsible for the maximal elimination rate is defined as the amount of injected triglycerides which per minute disappears from the total plasma olume (E mmoles/min)

2. *Th eff ctive flow f plasma passi g th active sites (the enzyme)* is defined as the amount of plasma which per minute is cleared from the substrate (F ml/min)

3. *Th ex gen lipid pa* is defined as the olume of distribution of the injected lipids (based on determinations of triglycerides in the top fraction of plasma separated in the PVP density gradient)

4 The plasma olume is defined as the ¹³¹I-albumin space.

Calculations

The obtained triglyceride concentrations, representing the exogenous lipids in plasma, were plotted against time on both arithmetic and semilogarithmic scales. K and k_2 were determined from the slopes of the curve obtained by ual fitting (Hallberg 1964—1965) The initial concentration (Ci mmoles/l) was obtained by extrapolating the arithmetically linea curv at high concentrations to zero time (see Fig. 3)

The amount of available enzyme activity (E) was calculated as K × plasma volume.

The effecti plasma flow (F) passing the acti sites was calculated as K_2 × plasma olume.

The lipid space was calculated by dividing the i jected dose (mmol) by Ci.

Statistical calculations were made (Snedecor 1957)

Results

Plasma vol me and l pid space

The plasma volume decreased during the noradrenaline infusion in all the dogs except three (Table I) The new volumes obtained seemed to be stable throughout the period of noradrenaline infusion, as judged from the plasma olume determinations performed in three dogs at the end of the infusion period (Table I)

The volumes of distribution of the injected lipids (the lipid pace) re given in Table I The lipid space was always greater than the plasma volume. There was a high correlation between the plasma olume and the lipid space (Fig 1) The correlation coefficient (r) was 0 996 before the infusion period and 0.997 during the noradrenaline infusion. The equ tions for the regression lines were y = 1 42X + 0 06 and y = 1 48X + 0.01 before and d ring the infusion respecti ely The slopes of the lines (1 42 and 1 48) were not significantly different

In three dogs (no. 5 11 and 12) the noradrenal ne nfusion caused rise in plasma olume (T ble I) In dog no 5 no rithmetically linea curve (K) was obtained, so the lipid space was not calculated. In the other two dogs the lipid space increased. The ratio between the plasma olume and the l pid space as the same as in th other dogs.

TABLE I. Body weight, plasma volume, and lipid space before and during noradrenaline-infusion in dogs. Doses of infused noradrenaline and intravenously injected fat emulsion before and during the noradrenaline-infusions

Dog no.	Weight kg	Noradrenaline γ/kg/min	Dose fat emulsion mmoles TG	Plasma volume L before	Plasma volume L during	Lipid space L before	Lipid space L during
1	13.4	0.11	5.56	0.83	0.79	1.27	1.18
2	16.0	0.38	6.20	0.92	0.68	1.37	1.01
3	15.5	0.29	6.67	1.00	0.82	1.50	1.23
4	6.8	0.44	2.67	0.63	0.38	0.95	0.57
5	13.2	0.23	5.10	0.76	0.81	—	—
6	17.0	0.35	6.63	0.86	0.79 (0.75)	1.32	1.14
7	10.3	0.44	3.50	0.63	0.47	0.92	0.73
8	6.8	0.66	2.67	0.42	0.37	0.72	0.59
9	11.7	0.39	5.55	0.71	0.58	1.04	0.82
10	9.2	0.39	3.34	0.49	0.38 (0.36)[1]	0.76	0.53
11	20.0	0.23	7.8	1.02	1.83	1.53	2.75
12	35.0	0.24	11.80	1.28	1.35	1.88	1.94

[1] Plasma volume determination at end of noradrenalineinfusion, i.e. after second dose of fat had been almost completely removed from the blood.

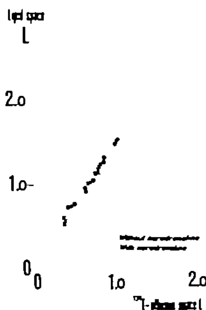


Fig 1 The correlation between the volumes of distribution of i injected fat emulsion (the lipid space) and ^{131}I-human albumin (the plasma volume) in dogs before and during an i infusion of noradrenaline at constant rate.

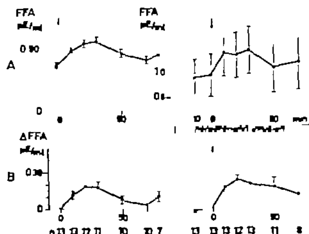


Fig. 2. A. The free fatty acid concentration (±SEM) in plasma in dogs given injection of fat emulsion (0.5 g/kg) before and during noradrenaline infusion at constant rate.
B. The individual changes in free fatty acid concentration (±SEM) in plasma in the dogs in A. n=number of dogs.

LLA

The basal level of LLA in plasma was very low and did not change during the noradrenaline infusion.

FFA

The first injection of the fat emulsion caused a transient rise of plasma FFA concentration from a mean basal level of 0.35 μ E/ml up to 0.55 μ E/ml 20—30 min after the injection (Fig. 2 A)

The noradrenaline infusion caused a rise in plasma FFA up to a mean level of 0.98 μ E/ml. The second injection of the fat emulsion caused a further rise up to 1 16—1 18 μ E/ml 10—30 min after the injection (Fig A) The standard error of mean of the plasma FFA during the noradrenaline infusion was larger than before the infusion, probably because of the variable FFA concentrations obtained with variable amounts of noradrenaline infused.

The standard error of mean of the individual FFA increases caused by the fat emulsion was the same before and during noradrenaline infusion (Fig 2 B)

Rate of elimination f injected fat em lsion

The results from a typical experiment re recorded in Fig 3 which shows the curves for the elimination of injected fat emulsion from plasma before and during the noradrenaline infusion.

It will be seen that the initial slope of the curv (K) during noradrenaline infusion is steeper than before. The initial concentration (C₀) is lower before than during the infusion. On the semilogarithmic scale the exponential part of the curve is steeper (K) bef re than during the infusion.

The obtained rate constants for the elimination are set out in Table II

The changes of the rat constants caused by the noradenal ne nfusion were the

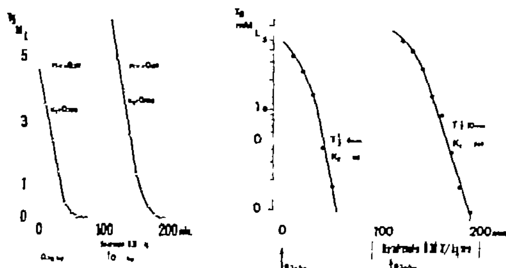


Fig. 3. The triglyceride concentration (on arithmetic and semilogarithmic scales) in the top fraction of plasma separated in polyvinyl-pyrrolidone density gradients versus time in one dog given an injection (at the arrows) of fat emulsion before and during infusion of noradrenaline at constant rate. The plasma volume (Pv) was determined before each injection.

TABLE II. Rate constants for elimination of fat emulsion from blood stream and calculated enzyme activity (E) and flow (F) (see definitions) before and during noradrenalineinfusion in dogs

Dog no	K mmoles/l/min		K l/min		E mmoles/min K_1 × plasma volume		F ml/min K_2 × plasma volume	
	before	during	before	during	before	during	before	during
1	0.100	0.113	0.099	0.082	0.085	0.089	84	6[illegible]
2	0.100	0.1 5	0.115	0.077	0.092	0.085	106	32
3	0.110	0.134	0.173	0.107	0.110	0.110	173	88
4	0.047	0.070	0.050	0.035	0.029	0.027	35	13
5	—	—	0.115	0.087	—	—	87	71
6	0.064	0.068	0.038	0.041	0.055	0.054	[illegible]0	32
7	0.083	0.120	0.050	0.069	0.054	0.056	31	32
8	0.050	0.060	0.087	0.087	0.021	0.022	37	32
9	0.085	0.104	0.046	0.026	0.060	0.060	33	15
10	0.094	0.120	0.030	0.030	0.046	0.046	25	11
Mean	0.082	0.102	0.084	0.064	0.061	0.061	66	41
P*		< 0.001		0.02	NS			< 0.02
11	0.130	0.080	0.087	0.099	0.134	0.146	89	181
12	0.096	0.087	0.024	0.0[illegible]0	0.123	0.118	31	67

* Degree of significance of the changes.

TABLE III Plasma volume, rate constants for elimination of fat emulsion from blood and calculated enzyme activities (E) and flows (F) before and after plasma transfusion in three dogs

Dog no.	Plasma transfused ml	Plasma volume L		K_1 mmoles/l/min		K_4 l/min		E mmoles/min K_4 × plasma volume		F ml/min K × plasma volume	
		before	after	before	after	before	after	before	after	before	after
13	150	0.41	0.52	0.076	0.048	0.115	0.087	0.031	0.025	47	43
14	100	0.47	0.55	0.050	0 042	0.063	0.069	0.024	0.023	30	38
15	300	0.50	0.87	0.140	0 076	0.095	—	0 070	0.066	47	—

same in all the dogs, excluding two (no. 11 and 12) with increased plasma volume. K decreased n dog no 5.

The rate constant for the maximal removal rate, K increased significantly and the fractional removal rate constant, K_2, decreased significantly (Table II) The reverse changes were observed in 2 dogs with increased plasma volume. There was a significant correlation (r=0.93) between the percentage change in plasma volume and the changes in K_1

Plasma transfusion

In the separate study of the rate of elimination of the fat emulsion before and after a plasma transfusion the increase in plasma volume determined with ¹ I-albumin was of the same magnitude as the transfused amount of plasma (Table III)

The maximal rate of elimination (K) of the fat emulsion decreased when the plasma volume increased (Table III)

The initial concentration (Ci) of the injected lipids in plasma decreased after the transfusion. The ratio lipid space/plasma volume after the plasma transfusion was the same as in the other dogs.

Calculation of E and F

The calculated E and F in the individual dogs before and during the noradrenaline infusion will be seen in Table II The infusion did not alter E but usually caused a decrease in F ($p<0.02$)

E did not change in the two dogs (no. 11 and 1) with increased plasma volume during noradrenaline F however increased in these dogs (T ble II) In dog no. 5, F decreased in spit of the increased plasma volume

In the three plasma-transfused dogs E and F were mainl nchanged (Tabl III)

The calculation of the mean of E per kg body weight ga e 0 0050 ± 0 0004 (SEM n=11) mmoles/kg/min.

Discussion

Definitions. The nature of rate constants in chemical processes are to be found in the atomic structure of the reacting materia (Glasstone *et al.* 1941). In biology and medicine the nature of metabolic rate constants are to be found in the structure of the living materia.

A great number of reactions are in vivo facilitated by enzymes. The differentiation of functions in vivo to different organs (organells) means that the different enzymes are *not evenly* distributed in the organism. There are places containing more substrate than the corresponding amount of enzyme and vice versa. The rates of the enzymatic reactions are thus dependent not only on the atomic structure of the reactants (enzyme and substrate) but also on the rate of transportation of the metabolites from one place to another. Metabolism may thus be characterized by the two events: the enzymatic rearrangement in the molecules and the transport of the metabolites.

Measurement of these two different functions independently must be of great clinical interest because it would be possible to differentiate between disorders in the enzymatic reactions and disturbances in the transportation of the metabolites.

Such measurements are difficult, however, because of a complex route of transportation between different compartments of unknown sizes and numbers. Assuming a) that a metabolite stays in plasma until it combines with the enzyme and b) that the target enzyme is localized in the surface of the vascular endothelium, such measurements would be possible in plasma.

Several lines of evidence suggest that chylomicrons and also the used fat emulsion are such a unique metabolite. The enzyme lipoprotein lipase is believed to be localized very close to the surface of the lumen of the vessels (*cf.* Cahill and Renold 1965). If a phagocytosis occurs (*cf.* Dole and Hamlin 1962) besides the process of hydrolysis of the TG by the enzyme, the engulfment process may also be considered as a saturable enzyme activity. The measurement of an enzyme is usually performed by measurement of its activity when the enzyme is saturated with substrate, i.e. maximal consumption of substrate per unit of time. This means that the reaction proceeds at a constant rate (mmoles/min), a zero order reaction by definition.

The definition of E (mmoles/min) in this report, as a measure of the available (enzyme) activity responsible for the elimination of the fat emulsion, is thus justified.

By multiplying the observed zero order (*lat. ce*) rate constant k_1 (mmoles/l *plasma*/min) by the plasma volume, the *absolute* rate, E, is obtained as an expression for the available (enzyme) activity in vivo.

A correlation between k_1 and heparin-induced LLA has been reported in dogs (Boberg and Hallberg). A transfusion or depletion of LLA in dogs influenced k_1 (Hallberg 1968). These two studies and others (*cf.* Dole and Hamlin) justified the consideration of E as a measure of the amount of available LLA responsible for the elimination.

An uneven distribution of the activity was confirmed in this study as no lipolytic activity (elimination in vitro) was found in the sampled blood, that contained the substrate. At low substrate concentrations, when the enzyme activity is not saturated the *flow* of substrates to the sites of activity will thus be a rate-limiting factor

The dog may consequently be considered as a system containing an unevenly distributed activity in contact with a flowing substrate. In such a system, at low substrate concentrations, the substrate will disappear at a fractional removal rate, the size of which is dependent on both the absolute flow (ml/min) and the volume of distribution of the substrate.

The rate constant K_2 (l/min) in this study is a *fractional* removal rate constant. By multiplying by the plasma volume the *absolute* plasma flow F is obtained.

In the definition of F the word effective was used because the calculated F may have no correspondence to physically measurable flows, but to the concept of clearance F thus, represents the sum of all small volumes of plasma containing the substrate which comes into contact with the active sites per unit of time.

Results

The infusion of noradrenaline caused a decrease in the plasma volume in most dogs, an increased zero order rate of elimination, K_1 and a decreased fractional removal rate, K_2. There were two dogs which reacted reversely in all these parameters. To confirm the two exceptions, the studies with the plasma transfusion were performed The results indicated that the changes in plasma olume were an important factor This justified the exclusion of the two dogs (no. 11 and 12) in the statistical calculations of the effect of noradrenaline (Table II)

The calculated lipid space was larger than the plasma volume. This may be due to rapid trapping of a part of the injected dose. It has been reported by Schoefl (1967 1968) that both chylomicrons and Intralipid stick to the vessel walls (see also Wasserman and McDonald 1963) This stickiness may explain the difference in apparent volume of distribution. A noradrenaline-induced reduction in the plasma vol ume is well known. This was confirmed in the present study However two dogs had a marked ncrease n the plasma volume This increase was accompanied by an increased lipid space The ratio between the lipid space and the plasma vol me was the same in all the dogs with and without noradrenaline This ndicates that the supposed tickiness was unchanged. The ratio was also independent on whether the plasma volume increased or decreased.

The FFA increase after jection of f t emulsion is probably referable to the hydrolysis of the TG in the emulsion (Wadström 1964) This ncrease was not influenced by noradrenaline. Thus there seems to be no competition between the ac tivity hydrolyzing the emulsion nd that responsible f th noradrenaline-induced change in FFA concentrations. However the change plasma olume nd changes in the fractional removal rates of FFA may mask portant changes.

In most of the dogs the rate constant K_1 incre d d ring the noradrenaline infusions. As noradrenaline is asoconstrictor nd hus ma exclud some of the

lipoprotein lipase activity from the circulation, it may seem contradictory that K_1 increased. This increase in activity will be clear if the dimension of the rate constant is considered. As K_1 is dependent on the plasma volume and as this volume usually decreased it is more relevant to measure the activity independent of the plasma volume. This is done by multiplying by this volume. So it was found that E was not influenced by the noradrenaline. It is thus justifiable to consider K_1 as a relative rate constant dependent on the plasma volume. The changes in K_1 in the studies with plasma transfusions give further support to this concept. As E did not decrease during the constant rate infusions, it is probable that the eventually induced vasoconstriction did not exclude from the circulation such enzyme activities as are concerned with the elimination of the fat emulsion from the blood stream.

A number of observations have demonstrated that noradrenaline reduces the blood flow through various tissues. The fractional removal rate constant K_2 decreased significantly (the two dogs with reverse changes excluded). The decreases are in accordance with a reduced flow of plasma passing the active sites.

The effects of surgical trauma on the rate constants K_1 and K_2 in man have been reported earlier (Hallberg 1965 b). Those observations showed that both K_1 and K_2 had increased 24 hours after abdominal surgery. The simulated trauma with noradrenaline in this study can only partly explain these observations. From the nature of the rate constants suggested here, the earlier observations may be explained either by an increased E or/and a decreased plasma volume. The observed manifold increase in K_2 may be explained either by a decreased plasma volume or/and increased F. The noradrenaline induced changes observed in most of the dogs may thus not explain the clinical K_2 changes. The observations in dogs no. 11 and 12 (Table II) are more in agreement with the clinical results.

Probably there are several other factors influencing K_1 and K_2.

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References

BOBERG, J. and L. A. CARLSON, Determination of heparin induced lipoprotein lipase activity in human plasma. *Clin. chim. Acta* 1964. *10*. 420—427.

BOBERG, J. and D. HALLBERG, Studies on the elimination of exogenous lipids from the blood stream. Effect of heparin on the elimination. To be publ.

CAHILL, G. F. and A. E. RENOLD, Regulation of adipose tissue metabolism within the intact organism: a Summary. *Handbook of Physiology sect. 5. Adipose tissue.* Washington. 1965. p. 682.

CARLSON, L. A. Determination of serum triglycerides. *J. Athero. scler. Res.* 1963. *3*. 334—336.

CARLSON, L. A. and D. HALLBERG, Studies on the elimination of exogenous lipids from the blood stream. The kinetics of the elimination of a fat emulsion and of chylomicrons in the dog after single injection. *Acta physiol. scand.* 1963. *59*. 52—61.

DOLE, V. P. and J. T. HAMLIN, Particulate fat in lymph and blood. *Physiol. Rev.* 1962. *42*. 674—701.

GLASSTONE, S., K. J. LAIDLER and H. EYRING, *The theory of rate processes.* International chemical series. Mc Grawhill Co Inc. New York 1941.

GOODMAN, L. S. and A. GILMAN, *The pharmacological basis of therapeutics.* MacMillan Co, New York, 3:e Ed, p. 476. 1958.

GORDON, E., Demonstration of tw kinds of fat particles in alimentary lipaemia with polyvinyl-pyrrolidone gradient columns. *Proc Soc p. Biol.* (N Y) 1962. *110* 657—661
HALLBERG, D Studies on the limination of exogenous lipids from the blood stream. Determination and separation of the plasma triglycerides after single injection of a fat emulsion in man. *Acta physiol scand.* 1964 *62* 407—421
HALLBERG, D Elimination of exogenous lipids from the blood stream. An experimental methodological and clinical study in dog and man. *Act physiol. scand* 1965 a. *65* Suppl. 254
HALLBERG, D Studies on the elimination of exogenous lipids from the blood stream. The effect of fasti g and trauma in man on the elimination rate of f t emulsion injected intravenously *A t physiol. scand.* 1965 b. *65* 153—163.
HALLBERG, D Transfusion of lipoprotein lipase activity *Act hir scand.* I prem.
H VEL, R. J A tonomic nervous system and adipose tissue. *Handbook of physiology sect 5 Adipose tiss* Washington 1965. p. 575.
RODBELL, M. and R. O SCOW Chylomicron metabolism, uptake and metabolism by perfused adipose tissue *Handbook of Physiology sect 5 Adipose tiss* Washington 1965 p. 491
SNEDECOR, G W *Statistical methods.* Iow State College Press, Iowa 1957
SCHOEFL, G I Report at the 11th Intern. Conf of the Biochemistry of Lipids, Jerusalem 1967
SCHOEFL, G L, Vascular permeability to particulate fat morphological observations on esrels of lactating mammary gland and lung. *Proc oy Soc B.* 1968. *169* 153—165.
TROUT D L E. H. ESTES J and S. J FRIEDBERG, Titration of free fatty acids of plasma study of current methods and new modification. *J Lipid Res.* 1960. *1* 199—204
WADSTRÖM, L Studies on the free fatty acid fraction in plasma during infusion of fat emulsion. *Act hir scand.* 1964 Suppl. 325.
WASSERMAN, F and T F McDONALD, Electron microscopic study of adipose tissue (fat organs) with special reference to the transport of lipids between blood and f t cells. *Z. Zellforsch Abt Histochem.* 1963 *59* 326—356.
WETTERFORS, J Catabolism and distribution of albumin in gastric cancer *A t med scand* 1962. *172* p. 163.

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Effect of Angiotensin on Sympathetic Nerve Activity

By

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Abstract

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The nervously mediated effect of angiotensin was studied in 13 rabbits by recording of renal and cervical sympathetic nerve activities, and was compared to results obtained with infusion of noradrenalin. I.v. infusion of angiotensin (0.5—3 μg/kg min) raised the blood pressure and initially reduced the normal rhythmical sympathetic activity. Whereas with noradrenalin the reduction of sympathetic nerve activity persisted, being determined by the blood pressure level, angiotensin shortly after the start of infusion was found to induce non-rhythmical activity in the renal nerve. This activity was associated with a further rise of blood pressure. The non-rhythmical activity and the concomitant pressure rise were transient, and disappeared after a few minutes, in spite of continued infusion of angiotensin. This suggests that the nervously mediated effect, although capable of causing considerable transient increase of arterial blood pressure, is of minor importance for the blood pressure response to prolonged infusion of angiotensin in rabbits. The non-rhythmical sympathetic activity was not related to changes in baroreceptor activity and was probably not caused by stimulation of chemoreceptors or sympathetic ganglions. Most likely this effect of angiotensin was brought about by interaction with central sympathetic neurons.

Recent experimental findings suggest that the vasoconstrictor action of angiotensin is partly nervously mediated. Perfusing the head of one dog with blood from another, Bickerton and Buckley (1961) and Buckley *et al.* (1963) found that administration of angiotensin to the donor and thus to the head of the recipient, caused a rise of systemic blood pressure in the recipient dog. Laverty (1963) found that infusion of angiotensin into the systemic circulation of rats caused vasoconstriction in the isolated vasculature of the hind limb. The response vanished after denervation of the limb. These investigators postulated that angiotensin induced sympathetic activity. We decided to examine this hypothesis by carrying out direct recordings of sympathetic nerve activity during angiotensin administration.

As a rise of blood pressure results in reflex reduction of vasoconstrictor activity, the

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effect of angiotensin on sympathetic nerve activity was compared to that of noradrenalin (NA). The nervous activity during administration of the two drugs was also recorded at normal pressure levels, obtained by bleeding the animals.

Material and methods

Experiments were performed on 13 rabbits (body weight 2,800—4,300 g) anesthetized with 3 ml 1 % chloralose and 3 ml 25 % urethane/kg: one-half to one-third of the total amount was given intravenously; the rest intraperitoneally. When necessary, urethane was supplemented during the experiments. The animals were tracheotomized and respired air. Respiration was not assisted. Rectal temperature was kept between 36 and 37.5° C using a heating lamp and covering towels. Arterial pressure was measured through a catheter in the right brachial artery with a Statham transducer. Systemic blood pressure could be varied by bleeding into and reinfusion from a reservoir connected to the right common carotid artery. Vasoactive drugs were infused through the right jugular vein.

The sympathetic and aortic nerves were dissected free on both sides of the neck and left intact. Left renal nerves were exposed through a lateral incision in the abdominal wall, the peritoneum being dissected free from the renal pedicle. The nerves were usually found as a distinct bundle firmly attached to the central side of the renal artery, and occasionally as several thin, separate nerves running in the loose tissue around the renal vessels. The nerves were prepared for recording by removing loose connective tissue in a length of about 10 mm, leaving the nerve sheath intact. Activity was recorded with two 0.4—0.5 mm thick platinum electrodes, 3—5 mm apart, connected to differential amplifiers. Quantitative measurements were obtained by rectifying and integrating the potentials, as previously described (Aars and Leraand 1968). The integrator had a long hold time, and was automatically discharged by a relay at a certain voltage. Neurograms from two nerves were recorded simultaneously and the integrated activity was recorded from one at a time by alternately switching the rectifying and integrating unit to the output from either of the two amplifiers. The neurograms, the integrated activity and the arterial blood pressure were recorded on a 4-channel jet ink writer (Elema Mingograph). Mean nervous activity was measured as the number of integrator discharges in a given time, usually 8 sec. The activity was calculated as per cent of activity at resting blood pressure, and each rabbit thus served as its own control. The electrical noise was usually negligible.

NA and angiotensin (Hypertensin CIBA) diluted in 5.5 % glucose were infused at a rate of 0.25, 0.75 or 1.5 ml/min. The concentrations were adjusted so that the applied doses of NA were 2—6 µg/kg min, and of angiotensin 0.5—3 µg/kg min.

Results

Activity in the renal nerves showed grouped discharges related to heart beats and respiration. The rise of arterial blood pressure caused by administration of NA resulted in a reduction of activity and usually in cessation of the firing above 120 mm Hg diastolic pressure. When the pressure rise was prevented by simultaneous bleeding, renal nerve activity remained unchanged. In the experiment illustrated in Fig. 1 NA was shown to cause a gradual reduction of sympathetic activity and almost abolition of activity at 125 mm Hg diastolic pressure. With angiotensin, the renal nerve activity showed the same response to pressure rise initially, but after about 20 sec considerable activity was recorded at the same pressure where NA had abolished all activity (Fig. 1, right panel).

Fig. 2 shows the effect of angiotensin on arterial blood pressure and cervical and renal sympathetic nerve activities in another rabbit. The blood pressure was increased to a new stable level. Sympathetic activity was initially reduced, but after about 10 sec a gradually increasing non-rhythmical activity was observed in the renal nerve. The non-rhythmical activity was accompanied by a further rise in blood

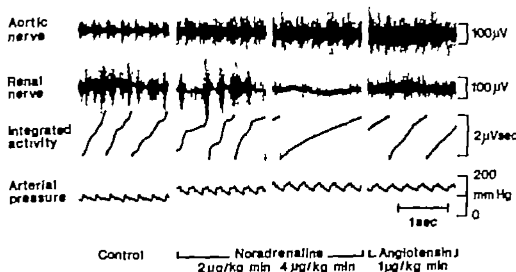


Fig 1 Effects of noradrenaline and angiotensin on aortic and renal nerv activities. Arterial blood pressure measured in brachial artery Renal nerv activ ty integrated. Activity recorded by the integrator during nfusion of 4 µg/kg min of noradrenaline was mainly caused by noise. The recording during administration of angiotensi was made 20 sec after start of the infusion.

pressure in this rabbit amounting to 20 mm Hg (Fig 2 D) After about 90 sec this non-rhythmical nervous activity began to diminish, in spite of continued infusion, and the blood pressure declined slightly Reduction of blood pressure by bleeding to r control pressure did not restore the non-rhythmical activity but permitted of about the same rhythmical activity as that observed before the infusion started.

Cervical nerve activity was initially reduced by the rise of blood pressure, although less than acti ity in the renal nerve. A secondary increase of acti ity as seen in the renal nerve was not observed in the cervical sympathetic nerve during administration of angiotensin

The variation with time of the non-rhythmical activity is illustrated in Fig 3 Renal nerve activity and diastolic pressure are plotted in periods of NA and angiotensin infusions in one animal NA produced a persistent drop in sympathetic activity A transient further rise of pressure due to increased infusion rate, was f l-lowed by even more marked depression of activity Infusion of angiotensin induced a similar pressure rise and initially a marked drop in renal nerve activity After 30 sec the activity began to increase, and this was accompanied by a further rise of pressure. A short period with increased infusion rate led to additional rise in pressure and acti ity the latter reaching 25 % of ctivity at control pressure. The non rhythmical activity diminished after 3—4 min, and could not be restored in this animal by increased dosage of angiotensin. In other rabbits a transient increase i activity could be demonstrated by increasing the infusion rate of angiotensin.

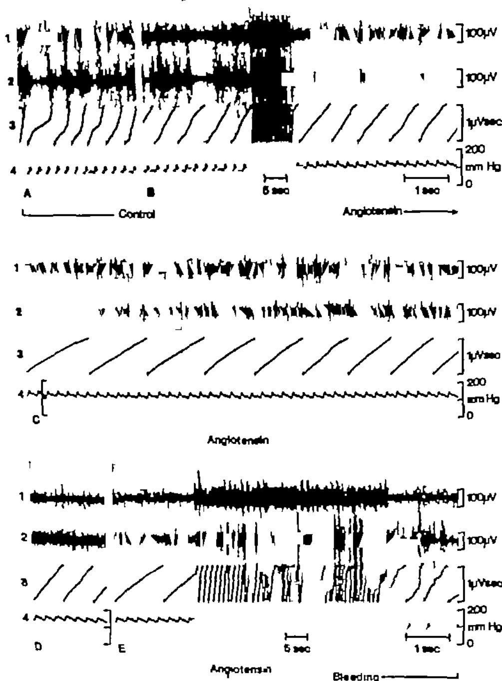


Fig. 2. Effect of angiotensin (1 µg/kg min) on sympathetic nerve activity (1) Cervical sympathetic nerve activity (2) renal nerve activity (3) integrated nerve activity and (4) brachial artery pressure. In recordings B and D cervical nerve activity was integrated, and in A, C and E, renal nerve activity was integrated. Small parts of the recordings were cut away (2 sec between B and C, 10 sec between C and D, 15 sec between D and E).

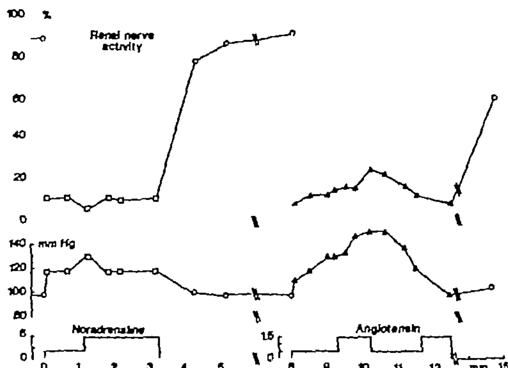


Fig. 3. Renal nerve activity and diastolic pressure during infusion of angiotensin and noradrenalin, related to time. Nerve activity as per cent of activity at control pressure after subtraction of noise. Doses of drugs in µg/kg · min.

○ without drug infusion, □ with noradrenaline, △ with angiotensin, ▲ during non-rhythmical renal nerve activity.

Renal nerve activity during infusion of angiotensin was examined in 11 animals and the non-rhythmical activity was repeatedly observed in all of them. It was never produced by NA alone but in two animals, the non-rhythmical activity was only observed when NA was given prior to angiotensin. The activity never exceeded 30 % of activity at control pressure. It started 6—200 sec after the initial pressure response, and usually lasted 40—120 sec. In some animals, the activity lasted as long as 4—5 min before ceasing. Maximal duration was not tested, however, in all rabbits. After a pause of a few minutes, non-rhythmical activity could be obtained again by a new administration of similar doses of angiotensin. Bleeding the animals to mean blood pressure of 30—50 mm Hg during infusion of angiotensin did not enhance the non-rhythmical activity, nor did it reproduce this activity after its spontaneous cessation. However, the presence of rhythmical activity, which was unaffected by angiotensin at normal and low blood pressures, made the non-rhythmical activity difficult to study in these situations.

The activity evoked by angiotensin was always accompanied by a second pressure rise of 5—30 mm Hg, which was closely related to the non-rhythmical activity both in time and magnitude.

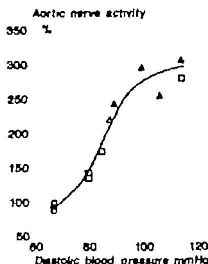


Fig 4 Relationship between diastolic pressure and aortic nerv activity during infusion of noradrenalin and angiotensin in one rabbit.
Symbols as in Fig 3

Activities in the renal and aortic nerves were simultaneously recorded in four rabbits. The relationship between arterial pressure and aortic nerve activity was unaltered in periods with angiotensin and NA infusion in two animals (Fig 4) In the other two rabbits, aortic nerve activity was higher with angiotensin than with NA at comparable pressures (Fig 1 right panels) but the pulsatile pattern of activity remained unchanged. The relationship between aortic and renal nerve ac tivities during infusion of angiotensin differed from that found with NA infusions only when non-rhythmical activity was observed In that case renal nerve activity was higher than expected from the aortic nerve activity

Electrical stimulation of aortic nerves (5—10 sec bursts of 60—120 pulses/sec. 0.1—1 msec duration, 6—8 V) had no effect on the extra non-rhytmical renal nerve activity but immediately reduced or bolished any normal rhythmical ac tivity

Cervical sympathetic nerve activity was examined in fou animals The acti ity was more continuous and less influenced by changes in pressure and aortic nerve stimulation than the activity in the renal nerve and adm nistration of ngiotensin failed to increase the activity

Activity in the distal end of the cut renal nerv a studied n f e animals and was found to be negligible and unaffected by f usi n of NA and angiotensin

Discussion

Infusion of NA and angiotensin was found to p od ce a rise of blood pressure and a subsequent initial reduction of rhythmical renal ne e acti t bu continued infusion of angiotensin induced a transitory non-rh thmical efferent n ty in the renal nerve This was not general ympathetic espo nse as no simultaneous in-

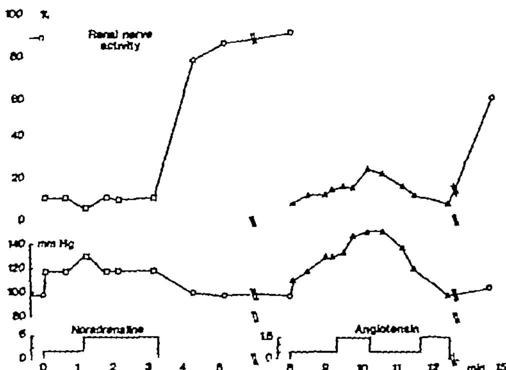


Fig 3 Renal nerve activity and diastolic pressure during infusion of angiotensin and noradrenalin related to time. Nerve activity as per cent of activity at control pressure after subtraction of noise. Doses of drugs in µg/kg min.
○ without drug infusion □ with noradrenaline △ with angiotensin. ▲ during non-rhythmical renal nerve activity

Renal nerve activity during infusion of angiotensin was examined in 11 animals, and the non-rhythmical activity was repeatedly observed in all of them. It was never produced by NA alone, but in two animals the non-rhythmical activity was only observed when NA was given prior to angiotensin. The activity never exceeded 30 % of activity at control pressure. It started 6—200 sec after the initial pressure response and usually lasted 40—120 sec. In some animals the activity lasted as long as 4—5 min before ceasing. Maximal duration was not tested, however in all rabbits. After a pause of a few minutes non-rhythmical activity could be obtained again by a new administration of similar doses of angiotensin. Bleeding the animals to mean blood pressure of 30—50 mm Hg during infusion of angiotensin did not enhance the non-rhythmical activity nor did it reproduce this activity after its spontaneous cessation. However the presence of rhythmical activity which was unaffected by angiotensin at normal and low blood pressures, made the non-rhythmical activity difficult to study in these situations.

The activity evoked by angiotensin was always accompanied by a second pressure rise of 5—50 mm Hg which was closely related to the non-rhythmical activity both in time and magnitude.

The effect of cutting the baroreceptor nerves could not be studied because the resulting profuse sympathetic activity masked the non-rhythmical activity. However Buckley *et al.* (1963) found that the effect of angiotensin persisted after baroreceptor denervation. Activity in chemoreceptors was not recorded in the present experiments, but the fluctuations in carotid chemoreceptor activity have been shown to persist also at high levels of activity induced by adding 5 % CO_2 to the inspired air or by clamping the common carotid artery (Biscoe and Purves 1967).

Angiotensin is known to stimulate sympathetic ganglia (Lewis and Reit 1965, Trendelenburg 1966) and also to facilitate ganglionic transmission (Haefely, Hürlimann and Thoenen 1966). Lewis and Reit (1965) observed a response in the nictitating membrane only with doses of angiotensin exceeding 0.1—0.3 µg into the artery supplying the superior cervical ganglion. Haefely *et al.* (1966) and Trendelenburg (1966) used doses of 1—3 µg angiotensin and the same application. These doses probably resulted in local concentrations of angiotensin far exceeding that obtained by the present intravenous dosage of 0.5—3 µg/kg/min in rabbits.

The present investigation confirmed that angiotensin will evoke sympathetic nerve activity. This effect of angiotensin was not caused by interference with the baroreceptors, nor was it probable that it was due to an effect on chemoreceptors. A direct ganglionic stimulation was ruled out by the comparatively small doses used. The most likely explanation, therefore, is that angiotensin acted by a direct or indirect stimulation of sympathetic neurons in the central nervous system.

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References

Aars, H. and S. Leraand, A method for quantitative analysis of aortic nerve activity. *J. appl. Physiol.* 1968. *24.* 416—418.

Ardaillou, O. O., W. C. B. Brown and W. I. Cranston, Pressor effects of angiotensin infusions into different vascular beds in the rabbit. *Clin. Sci.* 1966. *30.* 409—416.

Bickerton, R. K. and J. P. Buckley, Evidence for a central mechanism in angiotensin induced hypertension. *Proc. Soc. exp. Biol.* (N.Y.) 1961. *106.* 834—836.

Biscoe, T. J. and M. J. Purves, Observations on the rhythmic variation in the cat carotid body chemoreceptor activity which has the same period as respiration. *J. Physiol.* (Lond.) 1967. *190.* 389—412.

Buckley, J. P., R. K. Bickerton, R. P. Halliday and H. Kato, Central effects of peptides on the cardiovascular system. *Ann. N.Y. Acad. Sci.* 1963. *104.* 299—310.

Euler, [illegible] R., Aktionspotentiale der Nierennerven. *Arch. exp. Path. Pharmak.* 1957. *231.* 219—234.

Haefely, W., A. Hürlimann and T. Thoenen, *The effect of bradykinin and angiotensin on ganglionic transmission.* In Hypotensive polypeptides. Erdös, E. G., N. Back, F. Sicuteri and A. F. Wilde (Ed.). Springer Verlag, New York Inc. 1966.

Laverty, R., A nervously mediated action of angiotensin in anaesthetized rats. *J. Pharm. Pharmacol.* 1963. *15.* 63—68.

Lewis, G. P. and E. Reit, The action of angiotensin and bradykinin on the superior cervical ganglion of the cat. *J. Physiol.* (Lond.) 1965. *179.* 538—553.

Lockett, W. J. and A. E. Doyle, The pressor response to noradrenaline and tyramine during angiotensin tachyphylaxis in the dog. *Clin. Sci.* 1966. *31.* 247—254.

Sivalk [illegible] N., Problems in the morphology and nature of renal innervation. *Fed. Proc.* 1966. *25.* T 595—600.

Trendelenburg, U., Observations on the ganglion-stimulating action of angiotensin and bradykinin. *J. Pharmacol. exp. Ther.* 1966. *154.* 418—425.

Acta physiol. scand. 1968. 74. 142—152

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Effect of Direct Current on the Responses to Colored Flashes of Single Cells in Monkey Cortex

By

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Received 7 February 1968

Abstract

GULD C and M. LENNOX BUCHTHAL. *Effect of direct current on the responses to colored flashes of single cells in monkey cortex* Acta physiol. scand. 1968. 74 142—152

I monkey the response t monochromatic flashes was recorded of single units the cortex corresponding t with 1—2 from the fovea. All unit which responded selectively to blue t green or to red flashes did so in an *o* fashion.

Direct currents were applied through the tip of the record ng microelectrode The effects of currents were those to be expected of intracellular electrodes. Smaller polarizing currents than those exciting the cell directly affected the response to light. This effect was related to be cell' spectral sensitivity The response of red-sensitiv cells was decreased by depolarizing rrents but polarizing currents did not change the response of green-sensitiv units. The onclusion is that red- and green-sensitiv units have specific synaptic organization in the ortex. Units responding t all colors were affected by polarizing currents in the way to be expected, *i.e.* their response was increased by depolarizing and decreased by hyperpolarizing currents. The response of blu -sensitiv units was affected anously

De Valois and his co-workers described the responses of single cells in the lateral geniculate body of the monkey when the eye was flashed with diffuse monochromatic light (De Valois 1960 De Valois, Abramov and Jacobs 1966 and see for further references) Certain cells were color-coded" in that they responded to small dif ferences in wa e length, whereas there was little change in response when the light intensity was altered. These single cells responded to flashes across the visible spectrum, but oppositely to complementary colors that is *on* to blue, and *off* to yellow or *on* to green, and *ff* to red, or vice ersa. Wiesel and Hubel (1966) con firmed these findings in the main and demonstrated the spatial organization on which opposite responses to different colors is based Responses of the same kind were found in the visual cortex of the monkey in an area corresponding to 15 extra-foveally (Motokawa Ta a and Okuda 1962)

We used diffuse flashes and recorded from single cells in the monkey cortical area corresponding we believe to within 1—2 from the fovea. We did not find

opposite responses to opponent colors. Some cells responded to all monochromatic flashes and to white light other cells responded to a restricted portion of the spectrum, either to blue, or to green, or to red (Andersen, Buchmann and Lennox Buchthal 1962, Lennox-Buchthal 1965) We wished to explore whether the change in response pattern to monochromatic stimuli occurred in the geniculate or whether it was laid down by features of cortical organization. Our aims was to test both possibilities by supplying a current from or to the tip of the microelectrode used to record the responses to flashes of single cortical units. This was an approach suggested by W A. H. Rushton (personal communication)

Methods

Successful experiments were performed on fou adult monkeys, three sooty mangabeys (*Cercobus torquat at*)) and one vervet (*Cercopithecus ethiop pygerythrus*) weighing 6 to 10 kg The animals were anesthetized with Nembutal (36 mg/kg) initially and were then maintained under light anesthesia with chloralose 1 %—urethane 3 1/2 % The average maintenance dose was about 2 ml/kg s.c. every 2 hrs. The animals lay on thermostat regulated heating pad which maintained the rectal temperature t about 38 C They were given penicillin retard six and three days before the experiment and the respiratory tract was drained by gravity

The flash pparatus and the background illumination have been described in detail (Andersen *et al.* 1962) The only modification required by this study was the light source, which was Sylvania glow modulator tube type R 1130-C driven by 63 mA pulses from the output of square wav generator t rat of one flash every two seconds. The duration of the flash was usually 220 msec and was prolonged to 500 msec when necessary to establish whether late response was *on* or *off* Since the light source was weak, no more than 3 or 4 intensity steps, covering 2 decades, could be used. Maximum intensity of blue green and red flashes had the same quantal energy within 0.3 log units. Between experiments the light energy through each filter and t each intensity was checked by calibrated photo-cell by reference t calibrated tungsten filament bulb. The light was focused on the tropinized pupil. Recordings were from the contralateral cortex.

The electrodes (Wolbarsht, MacNichol and Wagner 1960 Guld 1964) recorded and delivered current extracellularly A platinum-iridium wire was sharpened lectrolytically coated with glass and platinated t the tip just before use. This type of electrode was strong enough to penetrate the dura. Units could be recorded from, in one tract, for as long as 24 hours and sometimes during second and third penetration, although the impedance rose in the dura and in the brain from the initial 2 to 5 MΩ to 20 t 50 MΩ. The advantages of recording by puncture through the dura *in situ* were that the brain moved less with respiration and pulsation, and that it did not become edematous and protrude through the craniotomy The disadvantage was that it was uncertain when the microelectrode entered the brain. Therefore the depth of penetration had t be expressed as μ below the first cell encountered

The direct curren was delivered through the tip of the recording microelectrode from a square wav generator and the artefact was neutralized by reference electrode over Wheatstone bridge. Current was directed outward or flowed inward from the tissue into the relatively negative tip of the microelectrode Current pulses were timed to start with the light and to outlast it by 200 to 300 msec the strength could be aried up to 10 A.

Potentials ere recorded via capacitance neutralizing amplifier (Guld 1962) and were displayed on one trace of an oscilloscope (Tektronix 565) Single sweeps (1 sec across the screen) were photographed with Grass camera. The impedance of the microelectrode was displayed on each sweep. In addition, each potential triggered the fast sweep (5 msec across the screen) and was displayed on it after suitable delay (Nissen-Petersen, Guld and Buchthal) A current corresponding to the used to trigger the light source and current corresponding to the direct current, were displayed on another beam. These currents indicated the duration of the flash and the strength, duration and direction of the current. The parameters of the stimuli (wave length, intensity and duration of the flash strength, duration and direction of the current) ere supplied in code numbers. This nformation as well as the latency (in msec of each potential were fed into an on-line computer and were written out on the bottom of each picture by four nixie-tubes The writing ne occurred during the second between flashes while the film was being transported between single sweeps. Artefacts ere identified by inspection of the enlarged pictures on the film and were excluded.

Procedure

A craniotomy was made over the area corresponding to central vision and the dura was spared. A plastic chamber was then cemented to the bone with dental cement and the head was fixed firmly by three stout metal pins driven into the cranium. The opening for the electrode was sealed with silicone grease, the chamber was filled with mineral oil from which bubbles had been removed by heat and suction, and the filling tube was clamped off to make an air-tight fluid-filled system (Davies 1956). The electrode was advanced in 1 µ steps (tallied by a counter) by a small motor placed in the lid of the chamber (larger model described by Andersen and Lauritzen 1959). When a unit was recognized by its spontaneous activity the motor was stopped and the contralateral eye was presented with flashes every other second through each of 15 monochromatic filters. The order of presentation was blue, green and red. Then flashes were delivered through a blue, a green and a red filter without direct current and with anodal and cathodal current. This series was repeated at maximum light intensity and when possible at lower light intensities. Control runs were interspersed when direct current was delivered without flashes, flashes without current, and blanks without flash or current. The current strength was usually about 1 to 2 nA. When possible, the threshold to direct current alone was sought, but increasing the current strength to 4 to 5 nA was apt to destroy the cell. Even when the current strength was kept at 1 to 2 nA, some cells deteriorated progressively with each positive or negative current series.

At the close of the experiment the brain was exposed and the position of the chamber and any visible electrode punctures were drawn on a map of the brain (Talbot and Marshall 1941).

Analysis of the data

For each unit, the number of potentials was counted at every 20 msec interval after the onset of the flash (or trace when there was no flash). The number of potentials per 100 flashes in each interval was calculated and the histograms of latencies were drawn (either by hand or by GIER computer) for each monochromatic flash without and with positive and negative direct current, for current without flashes, and for blanks. The mean histograms of Figs. 2 and 3 were constructed as the means of the number of potentials/20 msec/100 flashes of each unit. Only those units were included which were tested both by light and by positive and negative direct current.

Results

Effects of direct, extracellular current on 74 cortical units are described below.

Direct effects of locally applied current

Twenty three units were fired directly by the current delivered through the tip of the microelectrode used for recording. Fourteen of them had a hump on the ascending limb of the potential and were thus unequivocally cells. All potentials were initially positive and seven were almost entirely positive which indicated that the tip of the electrode was close to or possibly touching the cell membrane. About a third of the potentials had amplitudes of 3 to 6 mV and two thirds of 9 to 14 mV. Thus, almost no cells yielding potentials under 3 mV were fired (only one had an amplitude of 1 mV), but there were 20 cells with equally large potentials which nonetheless, did not fire directly in response to applied currents.

The discharge evoked by outward current (the tip of the electrode being positive with respect to ground) was an immediate burst (Fig. 1A). Repetition of the stimulus permitted the time of onset to be shorter, the frequency higher and the duration longer, presumably because the membrane had been rendered unstable. When the threshold could be determined it was on the order of 0.6 to 5 nA, and it was higher when the current strength was being increased than when it was being decreased.

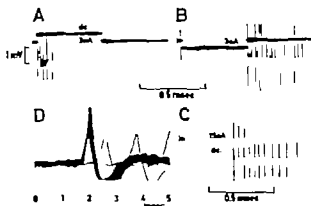


Fig. 1 To show the firing of cell by extracellular current (d.c.) delivered through the tip of the recording microelectrode. The current is displayed on the upper and the potential on the lower trace. Pass band 500 to 5000 cps.
A. Firing at the onset of positive current.
B. Firing at the break of negative current.
C. Negative current half the strength of the current in B the cell fires before the break of negative current as well as after it.
D. The same potential displayed on a fast sweep.

Similarly when a cell demonstrated damage by spontaneous or terminal firing, it could be fired by currents which previously had been ineffective. Eight cells disappeared within seconds after being fired by positive currents. In no instance was there a discharge at the cessation of an outward current.

The discharge evoked by inward currents, (the tip of the electrode being negative with respect to ground) was usually a break discharge (Fig. 1B and C) The cell was silent during the passage of current and discharged actively when the current ceased. Silence during the passage of current was less absolute towards the close than in the beginning of the stimulating time. Scattered potentials were seen as early as 200 msec after the onset of stimulation. Three cells fired during the flow of current towards the electrode and two of them also fired at the break of the current.

The direct effect of current is described to show that the cells reacted as if the microelectrode was intracellular Therefore, positive currents (at the tip) will be called depolarizing and negative currents (at the tip) will be called hyperpolarizing below

Effects of current on the response to light

Direct currents, smaller than those required to fire the cell directly could either accentuate the response to light or depress it. The current strengths ranged from 0.3 to 2 nA, *i.e.* about half the strength of current which fired the cell directly Cells whose response to light was affected by small currents tended to have smaller potentials than those fired directly in that the potentials of a third of the cells were under 3 mV while only a quarter of these units exhibited potentials of 9 to 20 mV Ninety per cent of the potentials had a hump on the ascending limb which indicated that they derived from cells, and all were initially positive.

The accentuation of the response to light could involve all parameters of the response *i.e.*, frequency latency threshold and spectral responsiveness, although not all parameters could be tested in each cells The spectral sensitivity could be

TABLE I. Effect of direct current on the response to flashes and the spectral sensitivity of single cortical units.

Effect on response to light	No.	Cell	Spectral sensitivity			
			Blue-sens.	Green-sens.	Red-sens.	To all
1. Incr. by pos., decr. by neg.	11	11	4			7 (1 off)
2. Decr. by pos., incr. by neg.	18	14	2		13 (72%)	1 (off)
3. No effect	19	17	5	13 (68%)	1	
Total	48	42	11	15	14	8

Hump on the rising phase of the potential.

broadened but the maximum response could not be shifted to a different spectral locus. That is, a unit which responded mainly to red might be brought to respond as well to green and blue but it could not be brought to respond solely to blue flashes and it still responded best to red. Only two cells gave stable *off* responses to light: the response of one was increased while the other was decreased by applied positive current.

In 11 instances the response to light flashes was accentuated by depolarizing and diminished by hyperpolarizing currents. Seven of these units had broad spectral sensitivity: six responding in an *on* fashion and one as an *off* to all colors (Group 1, Table I).

In 18 instances the effect of current was unorthodox in that the response to flashes was decreased by depolarizing and increased by hyperpolarizing currents (Group 2, Table I). Thirteen of the fourteen red-sensitive cells were affected in this way. The mean responses of these red sensitive units are drawn as histograms in Fig. 2. Without direct current, the response to red flashes reached its peak at about 100 msec after the onset of the flash. When the flash was accompanied by (up) positive (depolarizing) current, the *on* response was half as large as without current. This difference was noted at all light intensities and was judged significant by the chi square test ($p = 0.02$). When red flashes were accompanied by (up) negative (hyperpolarizing) current the initial peak of the response was the same as without current. However, the response continued to increase and the latter portion of the *on* response, (from 120 to 200 msec after the onset of the flash) was half again as large as without negative current ($p = 0.05$). The effect was most prominent when the flashes were weak, but this relation could have been fortuitous. The small *on* response of these red-sensitive units to green (shown in Fig. 2) and to blue flashes (not shown) were not significantly affected. Positive current without light fired no

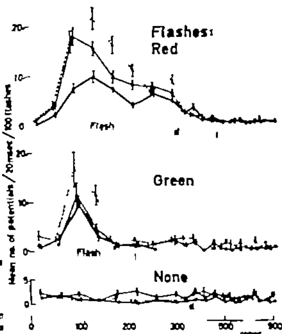
Fig. 2. *Histograms of latencies of 13 red-sensitive cells whose response to red flashes was decreased by positive and increased by negative direct current. The designations and the number of trials are*

Flashes	quanta/sec*	Direct current 1—2 nA None ○—○	Positive ●—●	Negative
Red	5.4 × 10[illegible]	1139	678	767
Green	8.4 × 10[illegible]	333	207	207
None		303	277	317

The construction of the histograms is described on p. 144 and p. 147

The vertical bars give the mean error

Intensities used in two thirds of the trials. Results with the lower intensities used were qualitatively the same.



cell one cell fired both at the make and at the break of a negative current without light (Fig. 2)

In 19 instances the response to light was slightly less during the passage of both positive and negative currents but the difference was not significant (Fig 3) Thirteen of the fifteen green-sensitive cells fell in this group (Group 3 Table I) The main double *on* response to green flashes and the small *on* responses to blue and red flashes were *similarly unaffected (Fig 3) irrespective of flash intensity Currents* with the same strength fired two cells directly

Only the responses of the red-sensitive cells in Group 2, Table 1 have been plotted in Fig. 2 to show how small the responses of these cells were to green flashes and that they were *on* as to red flashes (*small on responses to blue flashes not shown*) The main response to green flashes of the green-sensitive units in this group was halved by positive currents and the late *on* response was doubled by negative currents This same effect of current is illustrated in Fig 2 for the red-sensitive units responding to red flashes Only the responses of the green-sensitive cells of Group 3 (Table I) have been plotted in Fig 3 to show the small responses of these cells to blue and to red flashes, (5 units responded to the off as well as to the on of red flashes) The effects of current were the same as for the green-sensitive cells of Fig. 3 when the responses of all cells in Gorup 3 of Table I were plotted Those cells whose responses were recorded to several intensities of light were included in Table I and Fig 2 and 3 only if the effect of current was seen at all intensities The mean histograms at each intensity were the same as when responses to flashes of all intensities were massed as has been done in Fig 2 and 3 but, because of the small numbers the mean error was larger

The response to light of 12 units was affected erratically by extracellular currents even though two units responded to current directly

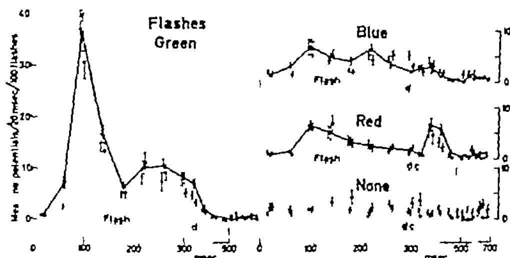


Fig. 3. Histograms of latencies of 12 green-sensitive cells whose response to light was unchanged by positive and negative direct current. The designations and the number of trials are:

Flashes	quanta sec	Direct current 1—2 nA None ○—○	Positive ●	Negative •
Green	8.4 10[illegible]	1270	713	636
Blue	7.4 × 10[illegible]	501	285	288
Red	5.4 10[illegible]	522	296	374
None		274	122	205

The construction of the histograms is described on p. 144 and p. 147.
The vertical bars give the mean error.

Intensities used in two thirds of the trials. Results with the lower intensities used were qualitatively the same.

Discussion

Spectral sensitivity and type of response

In this study as in our previous studies (Andersen *et al.* 1962, Lennox-Buchthal 1965) we recorded from single units in the visual cortex of monkeys. We believe these correspond, to judge by the cortical map of Talbot and Marshall (1941) and the description of Daniel and Whitteridge (1961), to within 1—2° from the fovea. Single units were found again which responded to all colors as well as units which reacted selectively to blue, green, or red. In this study selective responses to green and red were 1.5 to 2 times as frequent as responses to blue or to all colors (Table I).

In our previous study (Andersen *et al.* 1962) the flashes were brief and there was no certainty as to whether the response was at the onset of the flash or to its cessation. In the present study we used 220 msec flashes. These were usually long enough to allow the differentiation of *on* and *off* responses. When there was doubt as to whether a late response was *on* or *off* the flashes were prolonged to resolve

this question. Every cell that responded to a restricted portion of the spectrum did so in an *on* fashion. Small *x* responses were also noted to portions of the spectrum other than the cell's preferred locus. Thus, in the cortex corresponding to central vision, we have not located single cells giving opposite responses to complementary colors as do cells in the lateral geniculate body (De Valois 1960 De Valois *et al* 1966, Wiesel and Hubel 1966) and in the cortex corresponding to 15 extrafoveally (Motokawa *et al.* 1962)

Dir t effects of current flowing t a d from the tips of the xtrac llular microelectrodes used for r cording

Though the tip of the electrodes was extracellular the currents acted as if they were applied intracellularly (tip) positive currents gave *on* responses and (tip) negative currents evoked break discharges. McIlwain and Creutzfeldt (1967) reviewed the literature dealing with polarization with microelectrodes and added their own observations on the lateral geniculate body of cats. They called such recordings and effects of currents "quasi intracellular" Similar effects of local currents applied extracellularly were described in the hippocampus of rabbits (Euler and Green 1960) in the brain of frogs (Strumwasser and Rosenthal 1960) and rats (Bindman, Lippold and Redfearn 1962) and in the cat's visual cortex (Spehlmann and Kapp 1964) to select only those papers dealing with the brain. Most of these authors assumed that the tip of the electrode was in contact with the membrane of the cell. Svaetichin (1958) showed that currents, passed through an electrode in contact with the perikaryon of spinal ganglion cells, acted in the same way as i tracellular currents. Locally applied currents caused opposite ffects when the electrode tip was at some distance from the cell (Krnjevic and Phillis 1963)

Strumwasser and Rosenthal (1960) found excitation with extracellular positive currents of $1-3 \times 10$ A and spontaneous ctiv ty was modulated by currents one half to a third as large. Effective currents of the same size were reported by von Euler and Green (1960) and Spehlmann and Kapp (1964) whereas most effective currents were under 8×10^{-10} A In th study of McIlwain nd Creutzfeldt (1967) W often did not see direct effects of currents of this size on cells equally close to the microelectrode, judging by the size of the action potential. Perhaps the synaptic organization in the visual cortex of the monkey is such that the membrane of the cells is more stable than in the frog and the cat. It is tru that the polarizing currents w used were relatively brief (at most 500 msec) compared to the 3 to 10 second currents used by Strumwasser and Rosenthal (1960) and Spehlmann and Kapp (1964) But equally brief currents were effectiv n the studies of von Euler and Green (1960) and McIlwain and Creutzfeldt (1967) Prolonged currents, more than 5 min, produced effects lasting for hours (Bindman t al. 1962)

Effects of di t current n th esponse t l ght

The responses to light of cortical cells were modified by smaller currents than were required to excite the cells directly Th responses f about a quarter of the cells

were modified as reported by Spehlmann and Kapp (1964) Outward (tip positive) currents increased the discharge frequency of the response to light whether this was *on off* or *on-off* Since we found only few reproducible *off* responses, we can confirm this finding only with respect to *on* responses.

However the response to light of most of the cells was modified in the opposite way or not at all. The response of a third of the cells was diminished by outward (tip positive) currents. Since we used metal microelectrodes we could not record the membrane potential in the same cells. The phenomenon could be explained, however if a low membrane potential existed in these cells. Inactivation by depolarization was effected artificially in ventral horn cells by lowering the resting potential from —80 mV to —50 mV when antidromic stimulation could no longer evoke a spike discharge (Coombs, Eccles and Fatt 1955) A phenomenon thought to be spontaneous inactivation by depolarization was seen in the Purkinje cells of the cerebellum (Granit and Phillips 1956) Assuming such a condition for certain cells in the visual cortex of the monkey the response would be expected to be decreased by depolarizing currents. The latter portion of the *on* responses of the same cells was increased by hyperpolarizing currents.

The response of about a third of the cells was stable and was decreased only slightly by hyperpolarizing and by depolarizing currents. Probably synaptic organization in the cortex was responsible for this stability rather than defects in the method of supplying polarizing currents, since th same currents could affect the response of other cells giving equally large potentials. One defect in our technique was that the currents started with the flash and thus had, at most, 140 msec in which to affect the cell's *on* response to light. Since small currents may have to flow for seconds to change the cell's firing pattern (Strumwasser and Rosenthal 1960, Euler and Green 1960 Spehlmann and Kapp 1964) we might have seen effects of currents if they had flower longer before the response. This would not, however alter our conclusion that the response of about a third of the cells was remarkably stable

Spectral sensiti ity and effects of direct current on the e ponse t light

The spectral sensitiv ty of individual cells was, on the whole, constant. This sensitivity could be broadened somewhat during the flow of current but the main chromatic sensitivity was not altered. There was a clear relation betv een the spectral sensiti ity of the ingle cells and the effect of current on the response to light.

The responses of 13 of the 14 red-sensitive cells were decreased by depolarizing currents. This effect would be expected if the membrane potential of these cells were low Whether it was maintained at a low value by synaptic bombardment, or lowered by the flash or indeed, whether it was low at all, is an open question. The only suggestive evidence is that the effect on the red-sensitive cells was clear onl when the flashes were red—as if the red flash transmitted an effect as well a evoking a response. The responses of the few green-sensiti e cells affected in this way were decreased by depolarizing currents only when the flashes were green.

The responses of 13 of the 15 green-sensitive cells were stable during polarization. Both positive and negative currents insignificantly decreased the response. The *on* responses of these same cells to red and blue flashes were about a fifth as large as the *on* response to green flashes, and they too were slightly smaller during the passage of both positive and negative currents. Until there is evidence from intracellular recording, one can only speculate that the membrane potential of the green-sensitive cells is "locked" at a (presumably high and) steady level.

Whether or not the speculations indulged in are correct, our main conclusion seems justified, that is that red- and green-sensitive cells have a peculiar and specific cortical synaptic organization. Cells responding equally well to all colors do not seem to have a particular synaptic organization in the cortex. In any case nearly all of them were affected by polarizing currents in a predictable way, *i.e.*, their response to flashes was increased by depolarizing and decreased by hyperpolarizing currents. The responses of blue-sensitive cells were either accentuated by positive current, diminished by positive current, or unaffected by currents in no order of likelihood.

Blue-sensitive cells in the cortex were found only slightly less often than green- and red-sensitive cells, although blue *on*-center cells constitute 4 % of the units in the dorsal layers of the geniculate serving the contralateral eye (Wiesel and Hubel 1966). This fact, and the varied ways in which their response to light was affected by polarization, suggest that they must derive from geniculate cells of several different types, for example, from blue-*on* and from green-*on* cells (De Valois *et al.* 1966, Wiesel and Hubel 1966).

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References

ANDERSEN, V. O., B. BUCHTHAL and M. A. LENNOX-BUCHTHAL, Single cortical units with narrow spectral sensitivity in monkey (*Cercocebus torquatus atys*). *Vision Res.* 1962. 2 295—307.

ANDERSEN, V. O. and A. M. LAURSEN, Automatic transport device for microelectrodes. *Electroenc. ph. clin. Neurophysiol.* 1959 *11* 172—175.

BINDMAN, L. J., O. C. J. LIPPOLD and J. W. T. REDFEARN, Long-lasting changes in the level of the electrical activity of the cerebral cortex produced by polarizing currents. *Nature* 1962. *196.* 584—585.

COOMBS, J. S., J. C. ECCLES and P. FATT, The electrical properties of the motoneurone membrane. *J. Physiol.* (Lond.) 1955 *130* 291—325.

DANIEL, P. M. and D. WHITTERIDGE, The representation of the visual field on the cerebral cortex in monkeys. *J. Physiol.* (Lond.) 1961 *159* 203—221.

DAVIS, P. W., Chamber for microelectrode studies in the cerebral cortex. *Science* 1956 *129* 179.

DE VALOIS, R. L., Color vision mechanisms in the monkey. *J. gen. Physiol.* 1960 *43* 115—128.

DE VALOIS, R. L., I. ABRAMOV and G. H. JACOBS, An analysis of response patterns of LGN cells. *J. Opt. Soc. Amer.* 1966. 56 966—977.

Euler, C. von and J. D. Green, Activity in single hippocampal pyramids. *Acta physiol. scand.* 1960, *48* 95—109

Granit R. and C. G. Phillips. Excitatory and inhibitory processes acting upon individual Purkinje cells of the cerebellum in cats. *J. Physiol.* (Lond.) 1956, *133* 520—547

Guld C., Cathode follower and negative capacitance as high input impedance circuits. *Proc. IRE* 1962, *50* 1912—1927

Guld, C., A glass-covered platinum microelectrode. *Med. Electron. Biol. Engng.* 1964 *2* 317—327

Krnjević, K. and J. W. Phillis. Iontophoretic studies of neurones in the mammalian cerebral cortex. *J. Physiol.* (Lond.) 1963 *165* 274—304

Lennox-Buchthal, M. A., Spectral sensitivity of single units in the cortical area corresponding to central vision in the monkey. *Acta physiol. scand.* 1965 *65* 101—104

McIlwain J. T. and O. D. Creutzfeldt. Microelectrode study of synaptic excitation and inhibition in the lateral geniculate nucleus of the cat. *J. Neurophysiol.* 1967 *30* 1—21

Motokawa, K., N. Taira and J. Okuda, Spectral responses of single units in the primate visual cortex. *Tohoku J. exp. Med.* 1962, *78* 320—337

Nissen-Petersen H., P. C. Guld and F. Buchthal, A delay line to display random and evoked potentials. *Electroenceph. clin. Neurophysiol.* In press.

Spehlmann, R. and H. Kapp. Direct extracellular polarization of cortical neurons with multibarreled microelectrodes. *Arch. ital. Biol.* 1964 *102* 74—94

Strumwasser, F. and S. Rosenthal, Prolonged and patterned direct extracellular stimulation of single neurons. *Amer. J. Physiol.* 1960, *198* 405—413

Svaetichin G. Component analysis of action potentials from single neurons. *Exp. Cell. Res.* 1958, Suppl. 5. 234—261

Talbot S. A. and W. H. Marshall, Physiological studies on neural mechanisms of visual localization and discrimination. *Amer. J. Ophthal.* 1941 *24* 1255—1264

Wiesel, T. N. and D. H. Hubel, Spatial and chromatic interactions in the lateral geniculate body of the rhesus monkey. *J. Neurophysiol.* 1966, *29* 1115—1156.

Wolbarsht M. L., E. F. MacNichol Jr. and H. G. Wagner. Glass insulated platinum microelectrode. *Science* 1960, *132* 1309—1310.

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Muscle Spindle Responses to Concomitant Variations in Length and in Fusimotor Activation

By

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Abstract

LENNERSTRAND G and U THODEN *Muscle spindle responses to concomitant variations in length and in fusimotor activation* Acta physiol. scand. 1968. *74* 153—165.

The capability of the different fusimotor fibres to mediate the governing signals in the proposed "follow-up length servo regulation of muscle movements can be judged from the responses of spindle endings to increasing rates of fusimotor fibre stimulation concomitant with passive muscle shortening. Spindle endings in the ankle and the intercostal muscles have been studied. Dynamic and static fusimotor fibres have been stimulated in isolation or in combinations. Length and fusimotor inputs have been modulated in triangular waves, mostly of equal periodicity For servo action the afferent impulse frequency should be able to increase in pace with increasing fusimotor drive In spite of concomitant length decrements. Most of the static fibres, but none of the dynamic fibres, could induce an acceleration of the spindle discharge over the physiological range of length changes. This ability of the static fibres was not affected by the co-activation of dynamic fibre either with varying rates or with constant rate of stimulation, but when the activation by another static fibre was added, the increase in impulse rate was sometimes transformed into decline. Some static fibres, most of which had type II effects on the position sensitivity showed little ability to perform servo action as defined above Other functional implications of the results are also discussed.

It seems widely accepted that centrally demanded muscle movements are enforced both directly over the α motoneurones and indirectly through the external loop over the fusimotor neurones and the muscle spindles back to the α neurones (α—γ linkage of Granit 1955 see also Euler 1966) The balance between the two routes can probably be shifted, *e.g.* according to different demands on rate and force of muscle contraction (Hammond, Merton and Sutton 1956)

In motor control, the muscle spindle in conjunction with its fusimotor systems can be regarded as operating as a misalignment detector either in a length *regulating* system, compensating for external disturbances on muscle length or in a position servo mechanism, following a variable fusimotor input (Hammond, Merton and

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Sutton 1956, see also Milsum 1966) The capability of the spindles to serve in the length regulating function has been thoroughly worked out when analysing the responses to length changes during a constant fusimotor activation (*cf* Andersson, Lennerstrand and Thoden 1968*a*, also for references)

Servo function requires that the total afferent inflow from a muscle should have an increasing excitatory effect on the *a* motoneurones of the same muscle during the phase of contraction. This implies that the primary spindle endings, which are the only ones to make direct facilitatory contacts with the *a* motoneurones, should be activated by their fusimotor fibres so as to increase their afferent discharge against the unloading effect of contraction in the muscle to which they belong and thereby also enable them to compensate for the concomitant increasing inhibitory input from other muscle receptor afferents.

Results in support of the idea of a 'follow-up length' servo action have been supplied above all by the work on the respiratory muscles (*cf* Euler 1966, Sears 1966) For instance, the discharge rate of intercostal primary (and also secondary) endings has actually been found to increase during the contraction of the main muscle (Critchlow and Euler 1963, Euler and Peretti 1966) As far as the spindle is concerned, the prerequisites for a servo type of control would thus seem present.

The response characteristics of spindle endings to variations in fusimotor activity at constant muscle length have already been presented (Andersson, Lennerstrand and Thoden 1968*b* The present paper attempts to extend the servo aspects on spindle function by examining the spindle responses to concomitant variations in both length and fusimotor inputs. Such changes imply that an extension of the uscle is accompanied by a parallel decrease of stimulus frequency of the fusimotor isolated. It is known that both static and dynamic fusimotor neurones contribute to the rhythmic fusimotor activation of intercostal spindles (Euler and Peretti 1966 The prime object is to find out if the dynamic and the static fusimotor systems differ in their ability to promote servo control function, *i.e.* to accelerate the spindle discharge *in spite of muscle shortening*

During muscle lengthening in natural movements, *i.e.* in the phase of relaxation after contraction, the intercostal spindles discharged at a much lower rate than in contraction, indicating a rather abrupt diminution in fusimotor activation. The latter has been verified by recording from single *γ* fibres Eklund, Euler and Rutkowski 1964 Sears 1964 In some endings the discharge pattern in relaxation was identical to that of passive endings when the spindles were pharmacologically de-efferented (Critchlow and Euler 1963 Considering these facts, attention in the present study has been devoted particularly to spindle discharge patterns during muscle shortening in pace with increasing fusimotor excitation, while less care has been given to the response to length increment during decreasing fusimotor activation.

Spindle endings in ankle and intercostal muscles have been studied. Periodical length and fusimotor inputs of triangular wave form have been applied. Thus, when the repetition rates of both inputs were identical, as they mostly were, the frequency of fusimotor stimulation always increased in pace with the muscle shortening. It was

found that under the prevailing experimental conditions only static fibres were capable of increasing the spindle s firing rate during concomitant muscle shortening

However since this is a study of an open-loop system, the results cannot be used to elucidate the relative importance of the servo mechanism in the execution of motor action.

A preliminary report of some of the results has been published elsewhere (Lennerstrand and Thoden 1967)

Methods

The general procedures applied in the study of the hind limb and the intercostal muscle spindles hav already been presented (Andersson, Lennerstrand and Thoden 1968*a* Lennerstrand and Thoden 1968) In these papers also the mode of mechanical stimulation of the spindle endings was described. Periodic changes in the frequency of stimulation of single fusimotor fibres were accomplished in the way reported by Andersson, Lennerstrand and Thoden (1968*b*) Identification of fusimotor fibres was made from plots of impulse frequency against muscle length (*cf* Andersson, Lennerstrand and Thoden 1968*a*) The hind leg material comprised 6 dynamic fibres and 15 static fibres to 9 primary endings and 10 static fibres to 5 secondary endings. The intercostal material included 4 dynamic and 11 static fibres to 8 primary endings and 4 static fibres to 3 secondary endings. The numbers of static fibres of type I or II in each material are shown in Table I.

The amplitude of the changes of length was in the hind leg extensors 8 mm, in the hind leg flexors 5 mm and in the intercostal muscles 1.6 mm, which is roughly 10 % of their resting lengths. The range of variations in the stimulus frequency of fusimotor fibres was in all cases 60—180 pulses/sec. The mechanical and the fusimotor inputs were modulated along triangular waves. Most of the results were obtained with identical repetition rates of both inputs, t 1/2 or 1 /s. The matching of the two input signals 180 out of phase with one another was made by manual adjustments of the length signal generator Exact timing was hard to reach.

Results

In order to facilitate the description of th results, the concepts 'length response and 'fusimotor response ha e been introduced. Length response signifies the change in impulse frequency caused by variations in length t constant rate of fusimotor stimulation. 'Fusimotor response would be the change in impulse frequency to variations in fusimotor stimulation frequency with the spindle at constant length. For the purpose of comparing fusimotor effects, these responses may be expressed as the amplitude of the change in impulse frequency to a certain variation of length or fusimotor input. For instance, changes in the activation of primary end ngs by static f bres usually gave rise to larger fusimotor responses than did changes in dynamic fibre activ tion (Andersson, Lennerstrand and Thoden 1968*b*) For the length response during constant fusimotor stimulation frequency the effects tended to be the other w y round. However the discharge rate of n ending caused by simultaneous variations in fusimotor stim lation rate and muscle length will not be a pure summation of the length response and the fusimotor response since t each moment both of them are influenced by th alue of the other stim lus For this reason a quantitative description of the results would be ery comple nd is omitted. The results will instead by described in terms of a dominance of th length or of the fusimotor response and lead to a comparison with the discharge patt rn of impulses as seen n natural

Sutton 1956, see also Milsum 1966) The capability of the spindles to serve in the length regulating function has been thoroughly worked out when analyzing the responses to length changes during a constant fusimotor activation (*cf* Anderson, Lennerstrand and Thoden 1968*a*, also for references)

Servo function requires that the total afferent inflow from a muscle should have an increasing excitatory effect on the α motoneurones of the same muscle during the phase of contraction. This implies that the primary spindle endings, which are the only ones to make direct facilitatory contacts with the α motoneurones, should be activated by their fusimotor fibres so as to increase their afferent discharge against the unloading effect of contraction in the muscle to which they belong and thereby also enable them to compensate for the concomitant increasing inhibitory input from other muscle receptor afferents.

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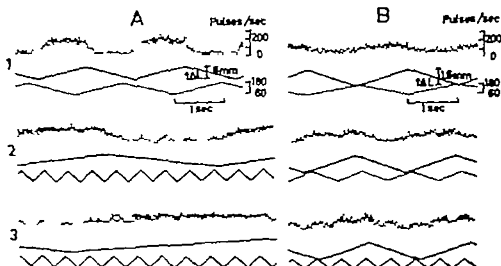


Fig. 2. Primary endings during concomitant triangular variation in length and in dynamic fusimotor activation of different repetition rates. Records as in Fig. 1. *A*, Flexor primary ending. *1* Repetition rate of length input = that of dynamic fusimotor input = 1/2 c/s. *2* Length = 1/4; fusimotor = 2 c/s. *3* Length = 1/8; fusimotor = 2 c/s. *B* Intercostal primary ending. *1* Length = fusimotor = 1/2 c/s. *2* Length = 1/2; fusimotor = 1 c/s. *3* Length = 1/2; fusimotor = 2 c/s. The length calibration represents 8 mm in *A* and 1.6 mm in *B*. Records retouched.

frequency of 120 pulses/sec. Thus, the length response of th primary ending gov erned the fusimotor response. This is illustrated in Figs. 1*A2* and 2 *A1* for leg endings and in Fig. 2 *B1* for an intercostal ending. However when the ratio of the repetition rates of muscle length input to that of fusimotor input was lowered to 1 2 in intercostal muscles and to 1 8 in leg muscles, which is equivalent to a reduc tion of the amplitude of the change of length, the spike frequency response d ring length decrement could follow the fusimotor signal (Fig 2 *A2* and *B2*) In order to obtain a clear dominance of the fusimotor response also in length increments still lower ratios of repetition rates were required 1 16 in hind limb primaries (Fig *A3*) and 1 4 in intercostal primaries (Fig. 2 *B3*) Part of the difference in ratios between leg and intercostal muscles could probably be accounted for by the differ ences in the absol te changes of muscle length applied to them (see Methods) When the velocities were about the same as in Fig 2 *A3* and *B3* the response characteristics were rather similar

In conclusion the dynamic fusimotor system could usually not induce the increase in spindle discharge *seen in muscle shortening of respiratory movements.*

Static fibres influence both primary and secondary endings (Appelberg, Bessou and Laporte 1966) When concomitant variations in static fibre stimulus frequency and in muscle length were similarly pplied to the spindles, a dominance of the fusimotor response over the length response was often encountered in both types of ending during length decrement and also during length increment (Figs. 1 *B2* and 5 *B*) However if the length response was relatively larger s in the ending of Fig. 3

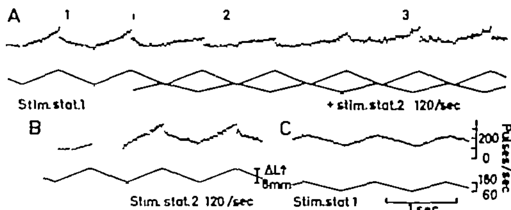


Fig. 3. Extensor primary ending (identified in *B*) activated by one or two static fibres. Repetition rate of triangular length and fusimotor inputs 1 c/s. Records as in Fig. 1. *A* Length changes. *1* During constant stimulation at 120 pulses/sec of static fibre no. 1. *2* During periodic variations in stimulus rate of the same fibre. *3* To the periodic static activation over fibre no. 1 is added stimulation at constant rate (120 pulses/sec) of static fibre no. 2. *B* Length changes, first in absence of fusimotor activation, then during constant stimulation at 120 pulses/sec of static fibre no. 2. *C* Variations in the rate of stimulation of fibre no. 1 at constant spindle length. Records retouched.

It could be seen to exceed the fusimotor response in length increment (Fig. 3 *A2*) in length decrement the fusimotor response was still the dominant. In this case the discharge rate fell rather abruptly when muscle stretch stopped and release of stretch started. Another type of fusimotor and length interaction is illustrated in Fig. 4. This from a secondary ending (*cf.* Fig. 4 *A*) under the control of a static fibre with [illegible] II effects on the position sensitivity (*cf.* Lennerstrand and Thoden 1968*b*). Length response (Fig. 4 *B*) surpasses fusimotor response (Fig. 4 *C*) and the combined action of varying both inputs together is a discharge pattern with dominant length response (Fig. 4 *D*).

In different muscles the two combinations of interaction occurred in different proportions, as shown in Table I. For example, the last mentioned type with a dominant length response also during length decrements was seen only in three intercostal

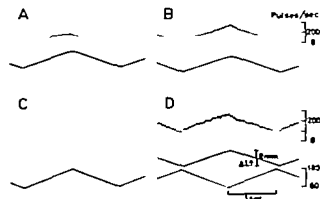


Fig. 4. Extensor secondary ending during static fibre stimulation. Records as in Fig. 1. *A* Length changes in the absence of fusimotor activation. *B* Length changes during constant static fibre stimulation at 120 pulses/sec. *C*. Variations in fusimotor activation at constant spindle length. *D* Length changes during periodic variations in static fibre stimulus rate. Repetition rates of triangular length and fusimotor inputs 1/2 c/s. Note differences in impulse frequency scale in *A—B* and *C—D*

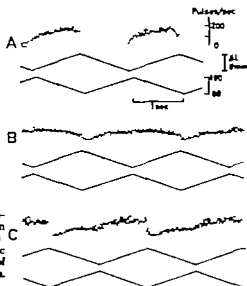


Fig. 5. Extensor primary ending during concomitant variations in muscle length and in the rate of stimulation of dynamic fibre (*A*) of static fibre (*B*) and of the dynamic-static fibre combination (*C*). Repetition rate of length and fusimotor input signals 1/2 c/s. Records as in Fig. 1 retouched.

endings but was very common in ankle extensor secondaries, which proved to be almost exclusively controlled by static fibres of type II action. The discrepancies between hind leg and intercostal spindles presented in Table I is likely to depend on differences in amplitudes and velocities of the length changes applied to the two sets of muscle. The combinations of the factors used above would lead to relatively larger length responses in leg muscle spindles. The results in Table I obtained with static fibres of type I and type II should be noted. During static type I activation of hind leg spindles a dominant length response during length decrement was seldom encountered, while during type II static activation this action was quite common, particularly in secondary endings.

Thus, static fusimotor activation was in most cases able to generate the increase in spindle activity during muscle shortening observed in natural movements. An attempt to interpret the meaning of the difference between static fibres of type I and II in their ability to perform this action will be made later on.

Co-activation of two fusimotor fibres. Under natural conditions spindle endings are most certainly concomitantly excited by several fusimotor fibres. It was therefore considered of importance to examine if the ability of static fibres to accelerate the discharge rate of an ending in muscle shortening was affected by the concurrent stimulation of other fibres to that ending.

As mentioned above both static and dynamic neurones rhythmically activate intercostal spindles during respiratory movements. When a dynamic-static combination was activated at varying rates of stimulation, the response to length decrement took exactly the course of the response during static activation alone (Fig. 5 *B* and *C*) whereas the response to length increment was slightly larger than that during dynamic single-fibre activation (Fig. 5 *A* and *C*). Both kinds of interaction are identical

TABLE I. The number of observations in which dominance of either the fusimotor response (fr) or of the length response (Lr) occurred in the impulse frequency responses of primary and secondary endings. Only the responses to length decrements concomitant with increasing rates of static fibre type I or II stimulation, are represented. Intercostal and hind limb spindles and static type I and II effects are presented separately

	Primaries		Secondaries	
	fr	Lr	fr	Lr
Intercostal				
Static I	5	0	1	0
Static II	5	1	1	2
Hind limb				
Static I	7	0	4	1
Static II	5	3	1	4

to those described for the length responses during the activation at a constant rate of a dynamic-static combination (Lennerstrand 1968). This interaction was explained as being due to complete occlusion of the dynamic fusimotor effect by the static action during length decrement and to some partial summation of the two single-fibre effects during length increment. In the present study such results have been obtained in 6 cases of dynamic-static co-activation. In the remaining combination the dynamic effect was weak and it was completely occluded by the static activation in all phases of the movement (see also Lennerstrand 1968).

During rhythmic static-static co-activation of primary and secondary endings the results varied more than in dynamic-static stimulation, just as was the case during fusimotor co-activation at constant rate. Partial summation of the individual discharge rates was observed in 4 out of 7 static-static combinations and complete occlusion in the rest. A separation into different modes of interplay as in dynamic-static combinations, depending on whether the ending was stretched or shortened, was not encountered during the stimulation of a static-static combination.

Only once in this rather small material of static-static combinations, a dominant fusimotor response during length decrement was changed into a dominant length response when another static fibre was rhythmically co-activated. Perhaps such interaction would occur proportionally more often in larger experimental series. It must be concluded, therefore that the rhythmic co-activation of one static fibre, yielding dominant length response during length decrement, could reduce the capability of another static fibre to accelerate spindle discharge during muscle shortening. On the other hand, the periodic concurrent stimulation of one dynamic and one static fibre appeared not to influence this ability of the static fibre.

Constant plus periodical fusimotor activation of a double-fibre preparation. In addition to the rhythmically active fusimotor neurones, intact intercostal spindles have

been shown to be excited by neurones discharging at a rather constant rate in all phases of respiration, so-called tonic fusimotor neurones (Critchlow and Euler 1963 Eklund, Euler and Rutkowski 1964) It was thought of interest, therefore, to find out how activation at a constant rate of one fusimotor fibre affected the response of a spindle ending to length changes concomitant with rhythmic fusimotor activation by way of another fusimotor fibre. Since both dynamic and static fibres are reported to be represented among the 'tonic fusimotor fibres (Corda, Euler and Lennerstrand 1966) both types have been tested as to their action as tonic fibres.

Figure 1 *A3* shows the influence of constant static fibre stimulation on the response of a primary ending to rhythmic dynamic activation. In Fig. 1 *B3* the dynamic fibre is activated at a constant rate and the static fibre periodically At both instances the dynamic effect dominated the response to length increment and the static effect the response to length decrement. During the latter phase the impulse freq ency curve is approximately the same as that obtained in static activation alone, whereas during length increment in combined stimulation the curve is shifted to slightly higher values than in dynamic single fibre activation. An explanation for this behaviour has already been proposed (see above and Lennerstrand 1968)

In Fig 3 is shown the response of a primary ending to length changes during the steady stimulation of a static fibre of type II effect (Fig 3 *B*) and the rhythmic activation of another static fibre to the same ending It is seen that the constant static activation reversed the slope of the impulse frequency curve to length decrement from a slightly positive (Fig 3 *A2*) to a definitely negative valu (Fig 3 *A3*) *i.e.* during the addition of a steady static activation there is a decline in discharge rate during muscle shortening During th added tonic activation by other static fibres causing less prominent length responses, the earlier acceleration of the spindle discharge in shortening persisted but was reduced in magnitude

Thus, 'tonic fibres with static effects, but not 'tonic dynamic fibres, could reduce the ability of other static fibres to increase spindle activity during length decrement.

Discussion

In the attempts to mimic the response pattern of spindle discharge obtained in natural respiratory movements it has been shown that waxing fusimotor activation of static fibres only but not of dynamic fibres, can increase the spindle s discharge rate during any appreciable length decrement. This difference between the two fusimotor systems was clearly discernable in hind limb spindles as well as in intercostal spindles. It should be observed that th length changes imposed on the latter were of about the same amplitude as those recorded in unrestrained respiratory movements (*cf* Andersson, Lennerstrand and Thoden 1968*a*) In order to increase the intercostal spindle discharge to length decrement during rhythmic dynamic fibre activation, the amplitudes of th concomitant length changes had to be reduced to less than one half of the original valu It has been certified that, w th regard to spindle responses both in the absence and the presence of fusimotor

tration, the passive length changes applied to a muscle are equivalent to active ones induced by varying the rate of motor nerve stimulation.

The range of stimulus frequencies (60—180 pulses/sec) is higher than that recorded from single intercostal fusimotor fibres during quiet breathing (4—140 pulses/sec, Eklund, Euler and Rutkowski 1964 20—100 pulses/sec, Sears 1964). It was tested in a couple of experiments that results similar to those presented could be obtained also within a lower range of stimulus frequency. The main reason for using a range of 60—180 pulses/sec was that strong fusimotor activation was wanted in order to obtain easily interpretable recordings. Thus, with reference to the statements in the Introduction, the static fusimotor system appears to take the largest share in the postulated servo control action exerted by the fusimotor activated spindles in aimed movements.

However not all static fusimotor single-fibres could evoke the response described above. Quite many fibres, most of which had type II actions on the position sensitivity were unable to cause an increase in the discharge rate during length decrement. Furthermore, the response pattern could be changed from an increasing to a decreasing discharge rate by adding other static fibres to the fusimotor input and stimulate them at a rate in pace with the original fibre or at a constant rate. In this connection it is of interest to note that, on co-activation with a static fibre a dynamic fibre never changed the single fibre effect of the static fusimotor fibre to length decrement. This observation is in agreement with earlier findings on dynamic-static co-activation at constant rate (Lennerstrand 1968) from these results it was suggested that the static and dynamic fibres influenced different intrafusal structures rather selectively and that the single fibre effect evoking the highest discharge rate in the ending partially or completely 'occluded' the action of the other fibre. In most cases this leads to purely static fusimotor effects on the primary endings during length decrement, while during length increment the dynamic fusimotor action is the dominant. This finding also implies that the filling-out of the pause in the spindle discharge to strong stimulation of the whole motor nerve (Matthews 1933) is mediated by static fibres.

In order to act as a 'follow up length servo' the sensitivity of the muscle spindle to outer disturbances, i.e. changes in muscle length, should be low relative to its sensitivity to the available range of variation in fusimotor activity. When the relation between the sensitivities is the other way round, as in primary endings under dynamic fusimotor control, a small operating range of length for the servo would be expected. However the servo system must be able to compensate adequately for outer disturbances like increase in load, in the manner prescribed by the 'load compensating reflex' of Corda, Eklund and Euler (1965) and therefore sensitivity to length changes is inevitably needed. The static fusimotor system has the required properties. Based on a similar conclusion, reached from studies on spindle behaviour to length changes during constant fusimotor activation only the static system has been termed the 'fusimotor trigger' system by Henatsch and Schäfer (1967). However as mentioned before, the static fibre group also contained

members which were unable to increase the spindle discharge in the manner required for a 'follow up length servo' Such static fibres, mostly of type II effects, appear instead to contribute mainly to the constant length control function governed by the spindle. This action Henatsch and Schäfer ascribed solely to the dynamic fibres, which were suggested to compose a 'fusimotor-sensor' system. From the present results it would seem as if the functional subdivision of the fusimotor fibres suggested by the authors quoted were an oversimplification. Moreover the secondary endings are not included in the scheme. These endings receive static fibre innervation. Their synaptic contacts in the spinal cord are only partly known, but in many cases they inhibit α motoneurones. Accordingly the general 'trigger' action of static fibres by way of secondary endings seems uncertain. In view of these objections we think that more extensive studies are needed on the fusimotor control of aimed and reflex movements, before a revision of the present fusimotor terminology is being considered. The provisional substitution of the terms 'dynamic and static' fibres for 'γ-trail' and 'γ-plate' fibres, respectively (Schäfer and Henatsch 1968) also needs some further evidence before it can be generally accepted. In fact, the only unquestionable correlation between fusimotor fibres and their effects has been obtained in fibres of mixed type (Adal and Barker 1965) though they have diameters in the α-range. These fibres terminate in 'plate' motor endings (Barker 1967). Nevertheless they induce dynamic fusimotor effects (Bessou, Emonet Dénand and Laporte 1965 Brown, Crowe and Matthews 1965)

In view of the functional differences between static fibres of type I and type II effects, revealed in the present study it might be permitted to speculate on the functional meaning of the earlier observed variations in the distributions of the two static actions to spindles in different muscle groups (Lennerstrand and Thoden 1968b) Fibres with type II action dominated in ankle extensor muscles, whereas type I effects were the most common ones in ankle flexor muscles. The extensor but not the flexor muscles, have a prominent postural function in addition to their role in limb movements (Denny-Brown 1929) This could imply that their spindles might be utilized for constant length control to a larger extent than the spindles in flexor muscles and that therefore the position sensitivity of the extensor spindles need be higher

Finally it should be mentioned that the two fusimotor systems seem to be brought into action separately in motor control. For example, stimulation of different structures in the central nervous system can evoke either dynamic or static fusimotor effects on the spindle response to stretch (Jansen and Matthews 1962, Appelberg and Emonet Dénand 1965 Appelberg and Molander 1967) and in the extensor rigidity of the decerebrate animal the static neurones seem the most active, while in the spinal animal the dynamic system is the leading one (Alnaes, Jansen and Rudjord 1965) In accordance with this the present results indicate that the static system is the dominant one in the co-activation of muscle spindles and α fibres in aimed movements.

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References

ADAL, M. N. and D. BARKER, Intramuscular branching of fusimotor fibres. *J. Physiol.* (Lond.) 1965. *177*. 288—299.

ALNAES, E., J. K. S. JANSEN and T. RUDJORD, Fusimotor activity in the spinal cat. *Acta physiol. scand.* 1965. *63*. 197—212.

ANDERSSON, B., G. LENNERSTRAND and U. THODEN, Fusimotor effects on position and velocity sensitivity of spindle endings in the external intercostal muscle of the cat. *Acta physiol. scand.* 1968a. *74*. In press.

ANDERSSON, B., G. LENNERSTRAND and U. THODEN, Response characteristics of muscle spindle endings at constant length to variations in fusimotor activation. *Acta physiol. scand.* 1968b. *74*. In press.

APPELBERG, B., P. BESSOU and Y. LAPORTE, Action of static and dynamic fusimotor fibres on secondary endings of cat spindles. *J. Physiol.* (Lond.) 1966. *185*. 160—171.

APPELBERG, B. and F. EMONET-DÉNAND, Central control of static and dynamic sensitivities of muscle spindle primary endings. *J. Physiol.* (Lond.) 1965. *63*. 487—494.

APPELBERG, B. and C. MOLANDER, A rubro-olivary pathway. I. Identification of a descending system for control of the dynamic sensitivity of muscle spindles. *Expl Brain Res.* 1967. *3*. 372—381.

BARKER, D., The innervation of mammalian skeletal muscle. In *Myotatic, kinesthetic and vestibular mechanisms*. Ciba Found. Symp. Editors A. V. S. de REUCK and J. KNIGHT. Churchill, London 1967. pp. 3—15.

BESSOU, P., F. EMONET-DÉNAND and Y. LAPORTE, Motor fibres innervating extrafusal and intrafusal muscle fibres in the cat. *J. Physiol.* (Lond.) 1965. *180*. 649—672.

BROWN, M. C., A. CROWE and P. B. C. MATTHEWS, Observations on the fusimotor fibres of the tibialis posterior muscle of the cat. *J. Physiol.* (Lond.) 1965. *177*. 140—159.

CORDA, M., G. EKLUND and C. von EULER, External intercostal and phrenic α-motor responses to changes in respiratory load. *Acta physiol. scand.* 1965. *63*. 391—400.

CORDA, M., C. von EULER and G. LENNERSTRAND, Reflex and cerebellar influence on α and on rhythmic and tonic γ activity in the intercostal muscle. *J. Physiol.* (Lond.) 1966. *184*. 898—923.

CRITCHLOW, V. and C. von EULER, Intercostal muscle spindle activity and its γ motor control. *J. Physiol.* (Lond.) 1963. *168*. 820—847.

DENNY-BROWN, D., On the nature of postural reflexes. *Proc. roy. Soc. B* 1929. *104*. 252—301.

EKLUND, G., C. von EULER and S. RUTKOWSKI, Spontaneous and reflex activity of intercostal gamma motoneurons. *J. Physiol.* (Lond.) 1964. *171*. 139—163.

EULER, C. von, The control of respiratory movement. In *Breathlessness*. Editors J. B. L. HOWELL and E. J. M. CAMPBELL. Blackwell Sci. Publ., Oxford 1966. pp. 19—32.

EULER, C. von and G. PERETTI, Dynamic and static contributions to the rhythmic γ activation of primary and secondary spindle endings in external intercostal muscle. *J. Physiol.* (Lond.) 1966. *187*. 501—516.

GRANIT, R., *Receptors and sensory perception*. Yale Univ. Press, New Haven 1955. p. 254.

HAMMOND, P. H., P. A. MERTON and G. G. SUTTON, Nervous gradation of muscular contraction. *Brit. med. Bull.* 1956. *12*. 214—218.

HENATSCH, H. D. and S. S. SCHAFER, Fusimotor-sensor and fusimotor-trigger functions. A re-interpretation of the dual control of mammalian muscle spindles. *Brain Res.* 1967. *6*. 385—387.

JANSEN, J. K. S. and P. B. C. MATTHEWS, The central control of the dynamic response of muscle spindle receptors. *J. Physiol.* (Lond.) 1962. *161*. 357—378.

LENNERSTRAND, G., Position and velocity sensitivity of muscle spindles in the cat. IV. Interaction between two fusimotor fibres converging to the same spindle ending. *Acta physiol. scand.* 1968. *74*. In press.

LENNERSTRAND, G. and U. THODEN, Responses of primary muscle spindle endings at constant or changing muscle length to variations in fusimotor activation. *Experientia* (Basel) 1967. *23*. 396—397.

LENNERSTRAND, G. and U. THODEN, Dynamic analysis of muscle spindle endings in the cat using length changes of different length-time relations. *Acta physiol. scand.* 1968a. *73*. 234—250.

LENNERSTRAND, G. and U. THODEN, Position and velocity sensitivity of muscle spindles in the cat. III. *Static fusimotor single-fibre activation of primary and secondary endings. Acta physiol. scand.* 1968b 74 30—49

MATTHEWS, B. H. C., Nerve endings in mammalian muscle. *J. Physiol.* (Lond.) 1933 *78* 1—33

MILSUM, J. H. *Biological control systems analysis.* McGraw-Hill, New York, 1966. p. 345

SCHÄFER, S. S. and H.-D. HENATSCH, Dehnungsantworten der primären Muskelspindel-Afferenz bei elektrischer Reizung und natürlicher Innervation der beiden fusimotorischen Fasertypen. *Exp. Brain Res.* 1968. 4 275—291

SEARS, T. A., Efferent discharges in alpha and fusimotor fibres of the intercostal nerves of the cat. *J. Physiol.* (Lond.) 1964 *174* 295—315.

SEARS, T. A., The respiratory motoneurone: integration at spinal segmental level. In *Breathlessness.* Editors J. B. L. HOWELL and E. J. M. CAMPBELL. Blackwell Sci. Publ., Oxford 1966. pp. 33—47

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Effect of Feeding on Antral and Duodenal Gastrin Activity

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Abstract

Fyrö, B. *Effect of feeding on ant al and duodenal gastrin activity* Acta physiol. scand. 1968. 74 166—172.

Antral and duodenal gastrin activity wa determined in cats at different times aft [illegible] meal of fish. Cat starved for 24 hrs were used s controls. At 1.5 and 3 hrs after feeding the antral gastrin activity wa reduced to about 40 per cent of controls At 6 hrs there wa [illegible] trend towards restoration and after 12 hrs the antral gastri activ ty wa back to control level. Starving for either 2 or 4 days did not influence the antral gastrin activity The duodenal gastrin activity was about 60 per cent of ontrols 3 hrs after feeding Vagotomy did not seem to influence the reduction of the gastrin activity 3 hrs after feeding.

Release of gastrin from the antral mucosa has been demonstrated indirectly by means of the acid secretory response from the oxyntic gland area of the stomach, to gastrin released by agal stimulation (Uvnäs 1942, Pe Thein and Schofield 1959) and by local mechanical (Grossman, Robertson and Ivy 1948) or chemical (Robertson *et al.* 1950) stimulation.

Recently another type of evidence for a release of gastrin was presented when reserpine (Emås and Fyrö 1965) and electrical vagal stimulation (Fyrö 1967) were shown to reduce the gastrin activity of the antral and duodenal mucosa of cat

The purpose of the present study was to investigate whether the gastrin activity in antral nd duodenal mucosa wa altered by a physiological stimulus like feeding which induces a agal as well as a local, stimulation of gastrin release

Methods

A total of 58 ca [illegible] 2.0 -4.0 kg fasted for 24 hrs, were used in the study They at a meal of about 200 g of raw f sh d ring 10 15 min and were killed 1.5 3 6 12 24 48 or 96 hrs later by an intraperitoneal injection of pentobarbital 300 mg/kg The [illegible] ts killed 24 hr after feeding were used as ontrols Vagotomized [illegible] ts were killed 3 hrs after the meal. The [illegible] ts starved for 48 and 96 hrs wer given 5 g of glucose in 100 ml of 0.9 % saline subcutaneously every 4 hrs.

The [illegible] gotomized [illegible] ts wer provided with gastric annulae (Emås 1960) Bilateral agotomy was performed a described by Emå 1964 The agotomy wa onsidered complet in all animals, since insulin hypogly emia produced no gastric acid secretion despite blood sugar level of less than 50 mg glucose per 100 ml blood during insulin hypogly emia The animals were used within 2 months [illegible] er agotomy

Preparation of Mucosa Specimens

The stomach and proximal duodenum were removed immediately fter death. Six hrs after feeding there was always some food left in the stomachs, whereas most of them were empty after 12 hrs. Food was found in the duodenum 1.5—12 hrs after the meal.

About 2 cm of the antral mucosa was stripped off 0.5 cm proximal to the pyloric sphincter i.e. well within the antral area. In some cats 4 cm segment of the duodenal mucosa was stripped off 0.5 cm distal to the pyloric sphincter The mucus was gently scraped off the specimens and the mucosa was stored at −20 C.

Preparatio of Gastrn Extracts

Gastrin was extracted according to the method of Komarov (1938) After standardization (Emås and Fyrö 1964) this method has shown atisfactory reproducibility (Emås and Fyrö 1964 and 1965) Each gastrin extract originates from the pooled antral or duodenal mucosa specimens of 2 cats. A series of 3—6 gastrin extracts were prepared simultaneously In every series 1 extract was derived from control cats. The number of gastrin extracts from non-agotomized cats at different times after feeding is given in Table I. From vagotomized cats, 2 antral and 2 duodenal extracts were taken three hours after the meal.

Ten extracts were randomly selected and assayed on guinea-pig ileum for histamine activity No histamine activity was detectable in any extract (<0.05 µg of histamine dihydrochloride per mg extract)

Assay of Gastrn Activity

The acid secretory activity of each antral gastrin extract was assayed on 4 non-anesthetized gastric fistula cats using histamine as the reference standard, as described by Uvnäs and Emås (1961) Due to the low secretory activity of the duodenal extracts, the amount of extract available allowed assay on only 3 cats. All extracts from series were assayed on the same animals. I all assay the dose of the antral gastrin extracts was 0.5 mg/kg and of the extracts from the duodenal mucosa 4 mg/kg

The gastrin activity was expressed in histamine units (HU) (Uvnäs and Emås 1961) per mg extract and in HU per g of mucosa. All alues were expressed as percentages of the mean control value in each series.

Evaluatio of Data

Data were analyzed in accordance with current methods for analysis of variance (Snedecor 1956) At each time after feeding, one comparison was made between the alues of all the gastrin extracts taken together and the control values. If there was no significant difference, the alues for each individual extract were compared with the corresponding control values. T evaluate the effect of vagotomy one comparison was made between the values for gastrin activity 3 hrs after feeding in non-vagotomized and agotomized cats in the same 2 series.

Results

Gastrn Extracts f om the Antral M cosa

The yield of gastrin extracts from the antra of control cats was 74 5 ± 5.2 (mean ± S.E. n = 9) mg per g mucosa and the mean secretory activity was 27 1 ± 5.9 HU per mg extract or 1960 ± 450 HU per g mucosa.

Effect of feeding on antral gastrin activity of n-vagot mized cats The mean yield of extracts (mg per g mucosa) from fed cats was not t any time significantly different ($p > 0.05$) from that of control cats (Fig 1)

TABLE I. Number of gastrin extracts from nonvagotomized cats at differen times after feeding

	1.5	3	6	12	24	48	96 hrs
Antral extracts	4	4	3	3	9	2	2
Duodenal extracts	1	2	—	—	2	—	—

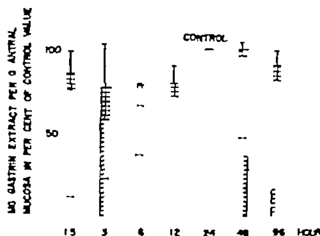


Fig 1 Mean yield of gastrin extracts per g antral mucosa of cats after feeding expressed as percentage of the corresponding control value. The 1.5- and 3-hr bars represent the mean yield of 4 extracts (4 groups of 2 cats) the 6- and 12-hr bars the mean of 3 extracts and the 48- and 96-hr bars the mean of 2 extracts. I = range

The secretory activity of the antral gastrin extracts (HU per mg extract) after feeding is shown in Fig 2. The mean secretory activity (4 extracts) 1 1/2 hrs after feeding was reduced to 47 per cent of corresponding controls. This change of secretory activity was statistically significant ($p<0.01$) Three hrs after feeding the mean gastrin activity (4 extracts) was reduced to 45 per cent of controls ($p<0.01$) Six hrs after feeding the mean activity (3 extracts) was 74 per cent ($p>0.05$) As this activity was not statistically different from controls, the activity of each gastrin extract, compared to the corresponding control, was analyzed individually The activity of the extracts was 42 ($p<0.001$) 67 ($p<0.01$) and 112 ($p>0.05$) per cent of controls, respectively Twelve hrs after feeding the mean value was 111 per cent and none of the 3 extracts was significantly different from controls. After starving for 2 and 4 days, the mean values for gastrin activity were 87 and 109 per cent, respec

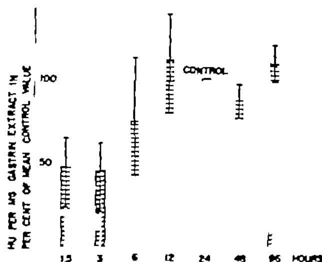


Fig. 2. Mean secretory activity per mg gastrin extract from the antral mucosa of cats after feeding, expressed as percentage of the corresponding mean control value The 1.5- and 3-hr bars represent the mean secretory activity of 4 extracts (4 groups of 2 cats the 6- and 12-hr bars the mean of 3 extracts and the 48- and 96-hr bars the mean of 2 extracts. I = range

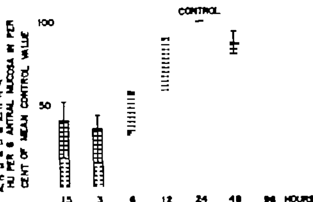


Fig. 3 Mean secretory activity per g antral mucosa of cats after feeding, expressed as percentage of the corresponding mean control value The 1.5- and 3-hr bars represent the mean antral gastrin activity of 4 extracts (4 groups of 2 cats) the 6- and 12-hr bars the mean of 3 extracts and the 48- and 96-hr bars the mean of 2 extracts. I = range.

tively None of the extracts differed (p>0.05) from the corresponding control values.

The antral gastrin activity (HU per g mucosa, Fig 3) ran almost parallel to the secretory activity of the gastrin extracts (HU per mg extract) since the yield of the extracts was fairly uniform and unaltered by feeding Th antral gastrin activity was reduced to 41 (p<0.01) and 36 (<0.01) per cent of controls after 1 1/2 and 3 hrs, respectively After 6 hrs, the mean activity was 58 per cent (p>0.05) The 3 individual values t this time were 33 (p<0.01) 53 (<0.01) and 88 (p>0 05) per cent. Twelve hrs after feeding the mean antral gastrin activity was 90 per cent (p>0.05) and only 1 of the 3 individual values was significantly different from controls (57 per cent p<0.01) After starving for either 2 or 4 days, the mean antral gastrin activity was 96 per cent and none of the individual values differed (p>0.05) from controls.

Eff ct of feeding on antral gastrin activity of vagotomi d cats The mean yield of extracts (mg per g mucosa) from vagotomized cats 3 hrs after feeding (2 extracts) was 90 per cent of controls. The secretory activity of the gastrin extracts was 41 per cent of controls mean of 39 (p<0.001) and 43 (p<0 01) per cent.

The mean antral gastrin activity (HU per g mucosa F g 4) of vagotomized cats 3 hrs after feeding was 38 per cent of controls in on series 28 (p<0.001) and in the other 47 (p<0 01) per cent. The corresponding mean value for non-vagotomized cats in the same 2 series of extractions was 29 per cent of controls in one series 16 (p<0 001) and in the other 42 (p<0 001) per cent.

There was no t tistically significant differenc (p>0 05) between the antral gastrin activity in gotomized and non-vagotomized cats 3 hrs fter feeding (Fig 4)

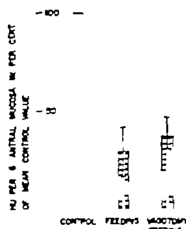


Fig. 4 Mean secretory activity per g antral mucosa of nonvagotomized and vagotomized cats 3 hrs after feeding, expressed as percentage of the corresponding mean control value. Each dotted bar represents the mean antral gastrin activity of extracts (2 groups of 2 cats) I = range.

Gastrin Extract from the Duodenal Mucosa

Gastrin was prepared from the duodenal mucosa in 2 series of extractions. The mean yield of gastrin extracts from the control cats was 59 mg per g mucosa and the mean secretory activity 1 4 HU per mg extract or 81 HU per g mucosa.

Effect of feeding on duodenal gastrin activity After 1 1/2 hrs the yield of extract mg per g mucosa) was 104 per cent of control (1 extract) Three hrs after feeding the mean yield of 2 extracts was 77 per cent in non-vagotomized and 89 per cent in vagotomized cats. The mean secretory activity was 44 per cent of control ($p<0.05$) after 1 1/2 hrs. Three hrs after feeding the activity for both extracts from non vagotomized cats was 75 per cent ($p<0.05$ and $p>0.05$ respectively) and for the 2 extracts from vagotomized cats 63 ($p<0.01$) and 67 ($p>0.05$) per cent of controls

The duodenal gastrin activity (HU per g mucosa) was 46 per cent ($p<0.05$) of the control value after 1 1/2 hrs. Three hrs after feeding the mean activity was 58 per cent of controls in one series 69 ($p<0.01$) and in the other 47 ($p<0.01$) per cent. In vagotomized cats the mean duodenal gastrin activity was 57 per cent of controls 3 hrs after a meal in one series 71 ($p<0.01$) and in the other 43 ($p<0.05$ per cent.

Discussion

Gastrin has been isolated from the antral part of the stomach of hog man, dog and sheep (Gregory and Tracy 1964 Gregory Tracy and Grossman 1966, Gregory 1966) and gastrin-like activity has also been obtained in extracts from the duodenal mucosa of cat, hog man and dog (Komarov 1938 1942, Harper 1946, Lai 1964 Emås and Fyrö 1965)

Experimentally induced changes in gastrin activity were first demonstrated by Emås and Fyrö (1965) who showed that reserpine reduced the activity in the antral, as well as in the duodenal mucosa of the cat. This effect was found to be blocked by vagotomy which did not *per se* alter gastrin activity indicating that vagal impulses were necessary for the reserpine induced reduction of the gastrin activity. It was suggested that reserpine induced central vagal activation, resulting in gastrin release. The recent demonstration that electrical vagal stimulation reduced antral and duodenal gastrin-like activity (Fyrö 1967) is compatible with this view and suggests that physiological alterations of vagal nerve impulse activity could induce changes in gastrin activity.

Feeding constitutes a physiological stimulus, which causes a vagal as well as a local stimulation of gastrin release. The present results demonstrate that feeding considerably reduces antral gastrin activity for at least 3 hrs. The duodenal gastrin-like activity also seemed to be reduced during the first hours after feeding. It seems reasonable to ascribe the reduction of the gastrin activity to a release of gastrin induced by feeding.

To exclude the vagal release of gastrin after a meal experiments were performed in which vagotomized cats were fed. Earlier results indicate that vagotomy *per se* has no effect on gastrin activity (Emås and Fyrö 1965) and in the present study vagotomy did not seem to influence the effect of feeding on gastrin activity either. Provided that the synthesis of gastrin is not influenced by vagotomy it is possible that intense local stimulation *per se* constitutes an optimal stimulus for gastrin release. However since vagotomy results in prolonged emptying time and a stasis of food in the stomach (see review by Alvarez 1948) it is possible that the local stimulation by the test meal is intensified, thereby masking the effect of vagotomy on the gastrin release mechanism. The effect of vagotomy on the gastrin activity after feeding could also be counteracted by a decrease in the acid inhibition of gastrin release, since the fundic glands are less sensitive to gastrin in a vagotomized stomach (Emås 1964, Emås and Grossman 1967) which might result in a lower acid response to feeding. In any case, the results of vagotomy and feeding indicate that local stimulation of the antrum can reduce the gastrin activity also after vagotomy.

In this study antral gastrin activity could not be reduced below about 30 per cent of the control level. This was also found in earlier studies, where no further reduction below this level could be obtained either by daily reserpine injections for 1—4 days (Emås and Fyrö 1965) or by electrical vagal stimulation for 2—4 hrs (Fyrö 1967). The gastrin activity remaining after vagal stimulation and feeding could represent a level at which the rate of synthesis balances the release. Alternatively there may be a pool of gastrin which cannot be released by vagal or local stimuli.

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References

ALVAREZ, W. C., Sixty years of vagotomy: review of some 200 articles. *Gastroenterology* 1948. *10* 413—441

EMÅS, S., Gastric secretory responses to repeated intravenous infusions of histamine and gastrin in nonanesthetized and anesthetized gastric fistula cats. *Gastroenterology* 1960. *39* 771—782.

EMÅS, S., Gastric acid secretion in gastric fistula cats before and after vagotomy and in vagotomized gastric fistula cats during reserpine treatment. *Acta physiol. scand.* 1964 *61* 255—271

EMÅS, S and B FYRÖ Antral gastrin activity in duodenal and gastric ulcers. *Gastroenterology* 1964 *46* 1—7

EMÅS S. and B. FYRÖ, Vagal release of gastrin in cats following reserpine. *Acta physiol. scand.* 1965 *63* 358—369

EMÅS S. and M I GROSSMAN, Effect of truncal vagotomy on acid and pepsin responses to histamine and gastrin in cats. *Amer J Physiol.* 1967 *213* 657—662.

FYRÖ, B., Reduction of antral and duodenal gastrin activity by electrical vagal stimulation. *Acta physiol. scand.* 1967 *71* 334—340

GREGORY R. A., Memorial lecture: The isolation and chemistry of gastrin. *Gastroenterology* 1966. *51* 953—959

GREGORY R. A. and H. J TRACY The constitution and properties of two gastrins extracted from hog antral mucosa. *Gut* 1964 *5* 103—117

GREGORY R. A., H. J TRACY and M. I. GROSSMAN, Isolation of two gastrins from human antral mucosa. *Nature* (Lond.) 1966. *209* 583

GROSSMAN M. I C. R. ROBERTSON and A. C. IVY Proof of hormonal mechanism for gastric secretion humoral transmission of distention stimulus. *Amer J Physiol.* 1948. *153* 1—9

HARPER, A A., The effect of extracts of gastric and intestinal mucosa on the secretion of HCl by the cat stomach. *J Physiol.* (Lond.) 1946. *105* 31P

KOMAROV S A. Gastrin. *Proc Soc exp Biol.* (N.Y.) 1938. *38*. 514—516

KOMAROV S A., Studies on gastrin. — II Physiological properties of the specific gastric secretagogue of the pyloric mucous membrane *Rev Canad Biol.* 1942 *1* 377—401

LAI, K. S Studies on gastrin. *Gut* 1964 *5* 327—341

PE THEIN M and B. SCHOFIELD, Release of gastrin from the pyloric antrum following vagal stimulation by sham feeding in dogs. *J Physiol.* (Lond.) 1959 *148* 291—305

ROBERTSON C. R K. LANGLOIS C. G MARTIN G. SLEZAK and M. I. GROSSMAN Release of gastrin in response to bathing the pyloric mucosa with acetylcholine *Amer J Physiol.* 1950. *163* 27—33

SNEDECOR, G. W *Statistical Methods*, Ed 5 Iowa State College Press, Ames, Iowa 1956.

UVNÄS, B The part played by the pyloric region in the cephalic phase of gastric secretion. *Acta physiol. scand.* 1942. *4* Suppl. 13.

UVNÄS, B. and S EMÅS A method for biologic assay of gastrin. *Gastroenterology* 1961 *40* 644—648.

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Vasopressin and Intrarenal Blood Flow Distribution

By

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Abstract

AUKLAND K. *Vasopressin and intrarenal blood flow distribution* Acta physiol. scand. 1968 *74* 173—182

It has been suggested that the rise in urine osmolality produced by antidiuretic hormone is wholly or partly due to juxtamedullary vasoconstriction with reduction of medullary blood flow This hypothesis was tested in 10 hydrated dogs in Nembutal anesthesia. Effective renal blood flow (*ERBF*) was estimated by p-aminohippurate clearance, and medullary blood flow by hydrogen gas clearance from the outer medulla measured polarographically with needle-shaped platinum electrodes. I. v. infusion of Pitressin, 3—120 mU/hr per kg body weight, or synthetic lysin-vasopressin, 2.5—30 mU/hr per kg, on average increased urine osmolality from 100 to 500 mOsm/kg Arterial blood pressure rose slightly with doses higher than 20—30 mU/hr per kg ERBF and outer medullary hydrogen clearance was not changed by 1—2 hrs vasopressin infusion, and also remained unchanged during return to water diuresis after stopping vasopressin. The experiments thus showed no effect of vasopressin either on total renal perfusion or on medullary blood flow

The generation and maintenance of high renal medullary osmolality is necessary for the production of osmotically concentrated urine S nce medullary blood flow tends to wash out solutes from the medulla, a lowering of medullary flow would be expected to increase the renal concentrating abil ty It has therefore been proposed repeatedly that one effect of antidiuretic hormone should be to reduce medullary blood flow by selective vasoconstriction in the juxtamedullary circulation Direct experimental evidence for this hypothesis is limited to a few observations Thurau, Deetjen and Kramer (1960) found that vasopressin infusion increased albumin transit time through the inner medulla of dogs, indicating a reduction of blood flow in this zone of the medulla. However they could not decid whether the ncreased transit time was due to medullary vasoconstriction or resulted from the rise n medullary osmolality with increased shunting of water from descend ng t ascending limbs of the vasa recta. It has also been shown, in dogs that dehydration lowers the rate of medullary uptake of Na^{24} (Levitin et al 1962) and Rb^{86} (Harsing and Pelley 1965) Since hydration and dehydration may influence the enal i culation by mechanisms other than changing endogeneous secretion of an d uretic hormone it would seem essential to study the effect of the hormone on medullary blood flow without changi g the degree of hydration In the present tudy this was accomplished by measuring

local hydrogen gas clearance from the outer medulla of anesthetized dogs with needle-shaped platinum electrodes, before, during and after intravenous infusion of vasopressin

Methods

The effect of vasopressin was investigated in 10 dogs of both sexes, weighing 17 to 30 kg. The animals had free access to water and on the day of the experiment 600—1000 ml water was given by stomach tube. 30—45 min later anesthesia was induced by Nembutal, 25 mg/kg i.v. and additional doses, 1—2 mg/kg were given, as needed, to maintain constant level of anesthesia throughout the experiment. Polyethylene catheters were placed in brachial artery for pressure recording and blood sampling, and in a brachial vein for infusions.

In all experiments 5 % glucose in water was infused at rate of 3 ml/min. Creatinine and sodium para-aminohippurate (PAH) were added in amounts giving plasma concentrations of 10—20 mg/100 ml and 0.5 to 1.5 mg/100 ml, respectively. To prevent glucosuria, crystalline insulin was added in concentration of 4 I.U./100 ml. Furthermore distilled water was given through the same catheter initially at rate of 4—6 ml/min, with reduction to 1—3 ml/min when a urine flow of 1.5 to 4.0 ml/min had been established. The infusion rate during the experimental period was thus 4—6 ml/min, and was not changed during or after vasopressin infusion.

The left or right kidney was exposed retroperitoneally through flank incision. The ureter was cannulated, and polyvinyl tubing of 0.5 mm external diameter was inserted into the renal artery in retrograde direction ad modum Herd and Barger (1964). Four or five platinum electrodes were tentatively inserted into the outer medulla, usually from the lateral aspects of the kidney as previously described (Aukland 1968). The active electrode tip had length of 1—2 mm, and a diameter of 0.3 mm at the base. The L-shaped electrode shaft was sutured to the renal capsule, the kidney returned to its normal position and the wound temporarily closed. Local hydrogen concentration was determined polarographically with polarizing potential of + 0.2 V versus calomel reference electrode, using six-channel polarograph and Rikadenki six-channel recorder (Rikadenki Kogyo Co., Tokyo, Model B-64). To provide reasonably good equilibration of hydrogen gas in the outer medulla and instantaneous desaturation of arterial blood, hydrogen gas was first given by inhalation for 5—10 min, and then maintained for another 1—2 min by infusion of hydrogen-saturated saline into the renal artery. The desaturation curves were plotted on semilogarithmic paper and local hydrogen clearance calculated as previously described (Aukland 1968, Aukland and Wolgast 1968).

position of each electrode was checked after the experiment on the excised kidney and electrodes in close contact with calyces or interlobar vessels were excluded. Of total of 39 electrodes anatomically positioned within the outer medulla, 5 were excluded for that reason. Two electrodes gave technically unsatisfactory desaturation curves, and 5 were excluded because of clearances less than 0.40 min based on the experiments reported below. The remaining 27 electrodes showed average clearance of 0.77 min in control periods.

Arterial blood pressure was measured with Statham transducer and recorded continuously on Sanborn recorder. Urine was collected in 10-min periods subsequent to determination of hydrogen clearance, arterial blood samples being drawn in the middle of each period. Creatinine in blood and urine was measured by the method of Bonsnes and Taussky (1945) and PAH according to Smith et al. (1945). Effective renal blood flow (ERBF) was calculated as PAH clearance divided by (1—Hct). Sodium and potassium were determined with Baird flame photometer and osmolality with an Advanced Instruments osmometer.

Each experiment started with control measurements during water diuresis, usually 3—6 measurements of outer medullary hydrogen clearance and 2 or 3 urine collection periods for determination of PAH, creatinine, sodium, potassium and total solute excretion. In one dog, only one control was obtained 15 minutes before beginning vasopressin infusion. Synthetic lysine vasopressin (LB-vasopressin) was given in 5 animals at rate of 2.5 to 30 mU/kg hr and Pitressin (Parke Davis & Co.) in 3 expts. in doses of 3—120 mU/kg hr. Pitressin is mixture of bovine and porcine pituitary extracts, thus containing both arginine and lysine vasopressin. Unfortunately no information could be obtained as to the fraction of each in the batch used in the present study. Vasopressin infusion was continued for 40—110 min with 2—8 clearance periods, and in five experiments the measurements were continued after stopping vasopressin infusion. The excised kidney was weighed after dissecting out the platinum electrodes and removal of perirenal fat.

Since urine flow per se might influence hydrogen clearance from the outer medulla, additional series of six experiments was performed in which urine flow was slowly increased over period of 1—2 hours by intravenous infusion of 5 or 10 mannitol. The clearances obtained from 17 electrodes in various positions in the outer medulla are shown in Fig. 1, where the lowest values were

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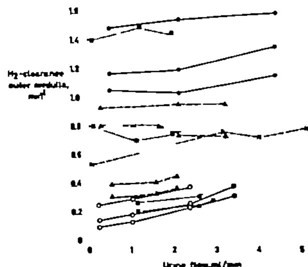


Fig. 1 Effect of mannitol diuresis on outer medullary hydrogen clearance measured with 17 electrodes in 6 dogs. Each dog represented by its own symbol.

obtained from electrodes at the border to the inner medulla, and the highest values close to the corticomedullary border (Aukland 1967) There seemed to be some increase in clearances with increasing urine flow at all levels of the outer medulla, but not in proportion to the control clearances at low urine flow Clearance from deep electrodes may in fact, show greater absolute increase than the higher clearances from more superficial electrodes. This may be due to the decreasing cross-sectional area of the renal medulla, going from the cortex towards the papilla. Since the net flux of water in this direction (= urine flow) is equal at all levels, the clearance due to urine flow might be expected t increase towards the papilla (Aukland 1967) Whatever the mechanism, clearance from the deepest layers of the outer medulla is considerably influenced by urine flow nd therefore unsuited for studies with large variations in urine flow Electrodes giving control clearances of less than 0.40 min were therefore excluded in the study of vasopressin effect.

Results

A detailed account of 2 expts. is set out graphically one with a large dose of Pitressin (Fig 2) and the other with a small dose of L8-vasopressin (Fig 3) Pitressin 120 mU/kg hr initially caused a marked rise of arterial pressure, followed by a transient return to control level. Urine flow fell rapidly from about 3 ml/min to 0.5 ml/min while urine osmolality increased from about 60 to 500 mOsm/l ERBF and outer medullary hydrogen clearance, as measured simultaneously with three electrodes, showed no consistent change.

The effect of L8-vasopressin, infused at a rate of 7.5 mU/kg hour is shown in Fig 3 The induced antidiuresis was not accompanied by any change in ERBF $\overline{AP}$ or outer medullary hydrogen clearance and unchanged values were also obtained n the post-infusion period In this experiment urine osmolality showed a transient rise in the control period Thus "spontaneous" antidiuresis possibly related to varying depth of anesthesia also occurred without changes in outer medullary hydrogen clearance or ERBF

Similar results were observed in all 10 expts. Since the effect of Pitressin and L8-vasopressin showed no obvious differences, all experiments hav been summarized in

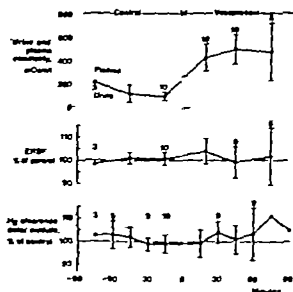


Fig 4. Effect of vasopressin infusion I. on urine and plasma osmolality, effective renal blood flow (ERBF) and outer medullary hydrogen clearance. Observations in 10 experiments grouped in 15 or 30 min intervals. Dots and vertical bars show mean ± 2SE for the number of observations indicated on the graph.

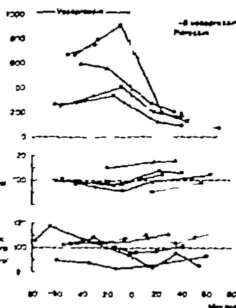


Fig 5. Urine osmolality, effective renal blood flow (ERBF) and outer medullary hydrogen clearance in 5 expts. during return to water diuresis upon stopping vasopressin infusion. Each dog represented by its own symbol.

small variations in these parameters were independent of each other or that any existing correlation was overshadowed by methodological errors in one or both determinations.

The infusion of vasopressin had no consistent effect on GFR. Urinary sodium excretion varied in control periods between 2 and 26 μEq/min and increased significantly during vasopressin infusion in all but 1 expt (Pitressin 3mU/kg hr) usually several times, to 25—196 μEq min. Potassium excretion, measured in 5 expts, also increased during vasopressin infusion but proportionally less than sodium excretion.

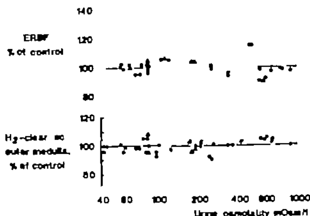


Fig. 6. Effective renal blood flow (ERBF) and outer medullary hydrogen clearance related to urine osmolality. 10 expts. Open circles: Control. Closed circles: Vasopressin infusion. Triangles: After vasopressin infusion.

Discussion

Three different mechanisms for the antidiuretic effect of vasopressin have been proposed (for references see Berliner and Bennett 1967). Vasopressin could 1) increase water permeability in the distal part of the nephron, 2) increase sodium reabsorption from the loops of Henle, or 3) reduce medullary blood flow. Whereas the first mechanism is well supported experimentally the evidence for mechanisms Nos. 2 and 3 is still indirect and equivocal.

The present experiments gave no evidence for a selective medullary vasoconstrictor effect of either lysine vasopressin or Pitressin. Arginine vasopressin which is probably the natural antidiuretic hormone in dogs, was not available in pure form. However the wide dose range of Pitressin, containing both arginine and lysine vasopressin should have exposed any medullary vasoconstrictor effect of any of these pituitary peptides. The conclusion would therefore seem valid for both lysine and arginine vasopressin. Before further discussion of the present experiments, it seems appropriate to consider previous data concerned with the role of medullary blood flow in the renal concentrating mechanism.

The fact that antidiuresis is induced by vasopressin in doses far below those required to increase arterial blood pressure or renal vascular resistance (References in Pickford 1966) does not exclude an effect on medullary blood flow since one could well imagine a much higher sensitivity to vasopressin in the juxtamedullary vessels than elsewhere in the circulation. On the other hand the extremely variable relationship between antidiuretic and pressor activity of various synthetic vasopressin analogues (Berde and Boissonnas 1968) is difficult to reconcile with this hypothesis.

The higher sodium concentration in the renal medulla and papilla in antidiuresis than in water diuresis (Ullrich and Jarausch 1956, Levitin et al. 1962) could result both from increased sodium reabsorption from the loops of Henle and from a lower medullary blood flow. However the difference might just as well be due to the diluting effect of a larger reabsorption of water from the collecting ducts in water diuresis than in hydropenia (Kill and Aukland 1960, 1961, Aukland and Kjekshus 1966). Selkurt and Wathen (1967) observed that Pitressin administration to squirrel

monkeys did not influence the washout of solutes from the renal medulla during mannitol diuresis. They concluded that "it seems highly unlikely that Pitressin has a constrictive effect on the medullary circulation out of proportion to its total effect on renal circulation" but again, the conclusion is based on the assumption that medullary solute concentration is mainly determined by blood flow. Moffat (1968) observed that 20 sec or more after i v injection of Thioflavin S labelled red cells the juxta medullary glomeruli contained fewer labelled cells relative to cortical glomeruli in hydropenia than in normally hydrated rats, and concluded that juxtamedullary blood flow was reduced in hydropenia. However since the normal vein to artery circulation time in rats is much less than 20 sec, the glomerular content of labelled cells will probably indicate the rate of removal and not the rate of uptake of red cells. Consequently if the observed difference has any relation to blood flow whatsoever it would rather indicate shorter juxtmedullary than cortical transit time in hydropenia, and accordingly higher medullary blood flow

A slower uptake of Na^{24} (Levitin *et al.* 1962) and Rb^{86} (Harsing and Pelley 1965) in the renal medulla in hydropenia than in water diuresis points more directly to a lower blood flow. However dehydration for 24—48 hr as used in their experiments, also produces marked reduction of cortical and total renal blood flow (Harsing and Pelley 1965) whereas antidiuretic doses of pitressin have no effect on total renal blood flow (Pickford 1966) It seems likely therefore, that the reduction of medullary blood flow observed by Levitin *et al.* (1962) and Harsing and Pelley (1965) was not mediated by antidiuretic hormone, but was rather due to other mechanisms brought into play by severe dehydration. Such a reduction of medullary blood flow might well explain the fact that higher urine osmolality is obtained by dehydration than by vasopressin infusion in normally hydrated individuals (Epstein Kleeman and Hendrikx 1957)

The unchanged PAH extraction in water diuresis and antidiuresis (Maxwell, Breed and Smith 1950) indicates, to some investigators, unchanged distribution of blood flow between cortex and medulla. It has been speculated, however that the variations might be too small to be detectable by this method (Selkurt 1963) which on the whole must be said to rest on unproven theories.

The finding of increased transit time for albumin through the inner medulla during pitressin infusion (Thurau *et al.* 1960) is repeatedly quoted as evidence for lowering of medullary blood flow in spite of the interpretation problems pointed out by the authors themselves. It has also been stated later that "In numerous unpublished experiments Kramer and associates could not demonstrate a vasoactive effect of ADH in physiological concentrations on medullary vessels" (Thurau 1964) This conclusion is in good agreement with the present study which showed no effect of vasopressin on either outer medullary clearance of hydrogen gas or on total renal blood flow. Provided hydrogen clearance reflects blood flow to the renal medulla, vasopressin thus did not influence medullary blood flow nor the distribution of flow between the cortical and the juxtamedullary circulation. The validity of local hydrogen clearance from the outer medulla as indicator for medullary blood flow has been discussed elsewhere (Aukland 1967 Aukland and Wolgast 1968) and will only

briefly be considered here. Since removal of hydrogen gas from the outer medulla by diffusion to the cortex is of small importance (Aukland 1967) there remains convective transport by blood flow in the vasa recta and urine flow in the loops of Henle and the collecting ducts. Volume flow in the loops of Henle would be expected to remain fairly constant in the present experiments, and is probably small compared to blood flow. As opposed to flow in the loops of Henle and in the vasa recta, the flow of urine in the collecting ducts is not opposed by an equal fluid flow in the opposite direction, and the clearance might therefore be considerably influenced by urine flow as previously observed for the inner medulla (Aukland and Berliner 1964). However, as judged from experiments with mannitol diuresis, the effect of urine flow is modest, except on clearance from the deepest layers of the outer medulla (Fig. 1) which were therefore excluded. Furthermore, the error introduced by varying urine flow would be to reduce clearance during vasopressin infusion and thus conceal a possible rise in medullary blood flow during vasopressin infusion. To the contrary, a possible reduction of medullary blood flow by vasopressin could be obscured by a concomitant reduction of kidney volume, or more specifically a reduction of the volume of the outer medulla. With unchanged absolute blood flow, the flow per volume tissue would increase, and clearance of hydrogen gas would also rise. However, Johansen, Kill and Loyning (personal communication) found by direct measurement of intrarenal distances that whereas hydration increased medullary volume considerably, infusion of Pitressin in hydrated animals caused practically no volume change. Similarly, Cross and Sherrington (1965) found that vasopressin caused no change of outer medullary water content in hydrated rats, whereas Levitin *et al.* (1962) observed a small decrease in dogs.

On the basis of dye injection studies, Moffat and Fourman (1963) pointed out that the outer medulla in rats receives blood from two sources: 1) through the vasa recta giving off capillary branches while traversing the outer medulla in the vascular bundles of this region, and 2) from the capillary plexus in the deep cortex through communications to the capillary plexus of the outer medulla. Fourman and Kennedy (1966) also observed that injection of pitressin in hydrated rats caused a reduced *staining* in the vasa recta, but not in the medullary capillaries, and concluded that vasopressin induced a selective constriction of the vasa recta. If vasopressin caused a similar redistribution of blood flow in dogs, it could conceivably reduce solute transport *through the outer medulla*, even in the face of unchanged total medullary blood flow. However, since the present study showed unchanged hydrogen clearance or unchanged "effective blood flow with respect to hydrogen", it seems unlikely that "effective blood flow with respect to sodium chloride or urea" should have been altered significantly.

A selective reduction of blood inflow to the inner medulla and papilla in the face of unchanged outer medullary blood flow seems unlikely because of the absence of vascular smooth muscle in the inner medulla (Moffat 1967). On the other hand, the present study does not exclude a reduction of volume flow reaching the papilla during antidiuresis, secondary to increased tissue osmolality and increased water shunting from arterial to venous vasa recta. Furthermore, it can not be decided

whether vasopressin stimulates reabsorption of sodium from the ascending limb of Henle's loops. The experiments indicate, however, that the increase in urine osmolality induced by vasopressin infusion in hydrated dogs is not caused by a selective vasoconstriction in the juxtamedullary circulation.

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References

AUKLAND, K., Study of renal circulation with inert gas measurements in tissue. *Proc. 3rd int. Congr. Nephrol.* Washington 1966. *1* 188—200. Karger, Basel/New York 1967.

AUKLAND, K., Effect of adrenaline, noradrenaline, angiotensin and renal nerve stimulation on intrarenal distribution of blood flow in dogs. *Acta physiol. scand.* 1968. *72.* 498—509.

AUKLAND, K. and R. W. BERLINER, Renal medullary countercurrent system studied with hydrogen gas. *Circulat. Res.* 1964. *15.* 430—442.

AUKLAND, K. and J. KJEKSHUS, Tubular salt and water transport in hydrated dogs studied with push-flow technique. *Amer. J. Physiol.* 1966. *210.* 971—979.

AUKLAND, K. and M. WOLGAST, Effect of hemorrhage and retransfusion on intrarenal distribution of blood flow in dogs. *J. clin. Invest.* 1968. *47.* 488—501.

BERDE, B. and R. A. BOISSONNAS, Synthetic analogues and homologues of the posterior pituitary hormones. In *The Pituitary Gland.* Ed. Harris, G. W. and B. T. Donovan. Butterworths, London, 1966. *III.* 624—661.

BERLINER, R. W. and C. M. BENNETT, Concentration of urine in the mammalian kidney. *Amer. J. Med.* 1967. *42.* 777—789.

BONSNES, R. W. and H. H. TAUSSKY, On the colorimetric determination of creatinine by the Jaffe reaction. *J. biol. Chem.* 1945. *158.* 581—591.

CROSS, R. B. and L. A. SHEREMETOVA, The effect of vasopressin on water distribution in the rat kidney. *Aust. J. exp. Biol. med. Sci.* 1965. *43.* 505—510.

EPSTEIN, F. H., C. R. KLEEMAN and A. HENDRIKX, The influence of bodily hydration on the renal concentrating process. *J. clin. Invest.* 1957. *36.* 629—634.

FOURMAN, J. and G. C. KENNEDY, An effect of antidiuretic hormone on the flow of blood through the vasa recta of the rat kidney. *J. Endocr.* 1966. *35.* 173—176.

HÅRSING, L. and K. PELLEY, Die Bestimmung der Nierenmarkdurchblutung auf Grund der Ablagerung und Verteilung von ⁸⁶Rb. *Pflügers Arch. ges. Physiol.* 1965. *285.* 302—312.

HERD, J. A. and A. C. BARGER, Simplified technique for chronic catheterization of blood vessels. *J. appl. Physiol.* 1964. *19.* 791—792.

KIIL, F. and K. AUKLAND, Renal tubular localization of water and sodium reabsorption in antidiuresis and water diuresis. *Scand. J. clin. Lab. Invest.* 1960. *12.* 277—289.

KIIL, F. and K. AUKLAND, Effect of high ureteral pressure on urine concentration during water diuresis. *Scand. J. clin. Lab. Invest.* 1961. *13.* 268—275.

LEVITIN, H., A. GOODMAN, G. PIGEON and F. H. EPSTEIN, Composition of the renal medulla during water diuresis. *J. clin. Invest.* 1962. *41.* 1145—1151.

MAXWELL, M. H., E. S. BREED and H. W. SMITH, Significance of the renal juxtamedullary circulation in man. *Amer. J. Med.* 1950. *9.* 216—228.

MOFFAT, D. B., The fine structure of the blood vessels of the renal medulla with particular reference to the control of the medullary circulation. *J. Ultrastruct. Res.* 1967. *19.* 532—545.

MOFFAT, D. B., Medullary blood flow during hydropenia. *Nephron* 1968. *5.* 1—6.

MOFFAT, D. B. and J. FOURMAN, The vascular pattern of the rat kidney. *J. Anat.* (Lond.) 1963. *97.* 543—553.

PICKFORD, M., Neurohypophysis and kidney function. In *The Pituitary Gland.* Ed. G. W. Harris and B. T. Donovan. Butterworths, London. 1966. *III.* 374—398.

SELKURT, E. E., The renal circulation. In *Handbook of Physiology.* Section 2. Circulation, Vol. II. Ed. W. F. Hamilton and P. Dow. Amer. Physiol. Soc. Washington, D. C. 1963. 1457—1516.

SELKURT, E. E. and R. L. WATERS, Renal concentrating mechanism of the squirrel monkey. *Amer. J. Physiol.* 1967. *213.* 191—197.

SMITH, H. W., N. FINKELSTEIN, L. ALIMINOSA, B. CRAWFORD and M. GRABER, The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. *J. clin. Invest.* 1945. *24.* 388—404.

THURAU, K., Renal hemodynamics. *Amer. J. Med.* 1964. *36.* 698—719.

THURAU, K., P. DEETJEN and K. KRAMER, Hämodynamik des Nierenmarks. II. Mitteilung. *Pflügers Arch. ges. Physiol.* 1960. *270.* 270—285.

ULLRICH, K. J. and K. H. JARAUSCH, Untersuchungen zum Problem der Harnkonzentrierung und Harnverdünnung. *Pflügers Arch. ges. Physiol.* 1956. *262.* 537—550.

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Aortic Baroreceptor Activity during Permanent Distension of the Receptor Area

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Abstract

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Aortic baroreceptor activity is known to be reduced in cases of persisting hypertension, the resetting probably resulting from changes in the aortic wall. The present investigation is an attempt to study the effects of permanent, localized dilatation of the aortic baroreceptor area on receptor activity. Ascending aortic stenosis was produced in 24 rabbits, and aortic diameters and aortic nerve activity were compared with results obtained in 10 normal rabbits. Aortograms four weeks after production of the stenosis showed diameters in the upper part of the ascending aorta and the aortic arch to be on average 16 % and 13 % greater than normal. Whereas an acute dilatation of this degree would have given marked increases of aortic nerve activity, the relationship between pressure and receptor activity was normal in animals with permanent dilatation, both at pulsatile and steady pressures. The load-length relationship of aortic strips from normal and dilated receptor area was also equal, indicating that the uniformity of the relationship between pressure and activity in the two situations, at least in steady states, was due to adaptation of the aortic wall. It thus seems that chronic increases of neither pressure nor receptor area diameter will have consequences for aortic baroreceptor activity.

Arterial baroreceptor activity is probably triggered by stretching of the arterial wall (Heymans and Neil 1958). The stretching is caused by the intraluminal pressure (P) which produces a tangential tension (T) in the wall (Laplace's formula, $T = P \times R$). The degree of stretching resulting from a certain pressure is thus determined by the wall tension caused by that pressure, and by the properties of the wall. The greater the radius of a vessel, the greater the tension produced by a given pressure.

In experimental hypertension where tension is primarily increased because of the permanently raised pressure, aortic baroreceptor activity has been found to be reduced, when compared to the activity in normal animals at equal pressures (Aars 1968 a). As this resetting of activity was associated with changes in the aortic wall, it was suggested that the resetting resulted from wall changes induced by the arterial hypertension (Aars 1968 b).

It is the object of the present paper to study the effect on aortic baroreceptors of permanently increasing the radius in the receptor area. The aortic baroreceptors are localized in and around the aortic arch, and diameter changes of this area were obtained by producing a post-stenotic dilatation. The dilatation, which is known to develop distal to arterial stenoses (Rodbard, Ikeda and Montes 1967) can be demonstrated 2—3 days after the induction of stenosis (Aars 1965, Roach 1963 a) and will reach a permanent state after a few weeks. Investigations of aortic baroreceptor activity were therefore made in one group of normal rabbits and in two groups of rabbits with ascending aortic stenosis of 3 days and 4 weeks duration respectively. Aortic nerve activity was studied during intact circulation, and also during artificial steady pressure perfusion of the aortic arch. The effect of the post stenotic dilatation on the properties of the aortic wall was assessed by subsequent *in vitro* studies of load-length relationships of circular strips from the receptor area.

Material and methods

The rabbits used for the experiments were of mixed breed, of both sexes with body weights ranging from 2100 to 4700 g. Ten animals served as normal controls. Aortic stenosis was established in 24 animals. Ten of these were examined 3 days (3 D) after, the remaining fourteen 4 weeks (4 W) after establishment of the stenosis.

Aortic stenosis was produced by constricting the ascending aorta with a nylon ring. For this operation, the animals were anesthetized with sodium pentobarbitone (Nembutal) 30 mg/kg body weight [illegible] and ether. They were intubated and connected to a positive pressure respirator (Aars and Kristiansen 1963) which delivered various mixtures of air and ether. The chest was opened in the second or third intercostal space. A nylon ring, with inner diameter of 3—3 mm, was cut in two and tied together around the aorta about half way between the heart and the brachio-cephalic trunk (Aars 1965).

On the day of nerve recording, the animals were anesthetized with a mixture of 5 ml 1 % chloralose and 3 ml 25 [illegible] urethane per kg. One-half to one-third of the total amount was given intravenously, the rest intraperitoneally. When necessary, urethane was supplemented during the experiment. Arterial pressure was measured in the right common carotid artery with a Statham transducer. The right jugular vein was cannulated for withdrawal and infusion of blood and other intravenous infusions. Clotting was prevented by [illegible] administration of 500—1000 I.U. of heparin. The degree of dilatation was checked by aortography prior to the nerve recording. Between 4 and 5 ml 60 [illegible] Isopaque (Nyegaard & Co A/S, Oslo) were injected through the right jugular vein by means of a syringe operated by compressed air at 3 kg/cm². The injection triggered the first exposure and all exposures were marked on the simultaneous running pressure recording (Fig. 1 C). About 6 exposures of 2—4 frames/sec were made with monoplane film-shifter. A Vernier gauge which allowed readings to nearest 0.1 mm was used for measuring the following aortic diameters on the films (Fig. 1 A, B): Lower ascending (LASC), upper ascending (ASC), aortic arch (ARC) and 3 cm down the descending aorta (DESC). The results were expressed as the ratio LASC/DESC, ASC/DESC and ARC/DESC for each animal.

For recording of aortic nerve activities, left and right aortic nerves were dissected free in the neck, and whole nerve activity was recorded by means of platinum electrodes and a differential amplifier (Tektronix 1[illegible]). Quantitation of activity was obtained by rectification and integration of the potentials (Aars & Leraand 1968). The full and rectified neurograms, the integrated activity and the arterial pressure were recorded on a 4-channel jet ink writer (Elema Mingograph) with a paper speed of 50—100 mm/sec. The nerve activity was recorded at various diastolic blood pressure levels obtained by stepwise withdrawal and reinfusion of blood. Measurements of mean nerve activity was normalized by calculation to mean activity per second at a heart rate of 300 beats/min. Systolic nerve activity was measured during the first 0.04 sec of systolic pressure rise and diastolic activity [illegible] the last 0.04 sec immediately prior to the pressure rise (Aars 1968). The measured activities were grouped in 10 mm Hg steps and their mean values were calculated for each animal and for each group of animals.

Left aortic nerve activity was successfully recorded [illegible] nine normal rabbits, eight 3D and fourteen 4W rabbits.

Recordings of right aortic nerv activity were obtained in seven 3D rabbits, eight 4W and fiv normal rabbits.

I most of the animals attempts were made t record aortic nerve activity during perfusion of the aortic arch. This was done in order to standardize receptor stimulus, and in orde to obtain records of aortic nerv activity under conditions comparable to the later *in vitro* load/length studies of the aortic wall. The thoracic cavity was opened wide with the rabbit flat on its back. Ventilation was maintained with the respirator. Blood was drained either from the lef atrium into reservoir or from the right atrium and right entricle into an oxygenator (Fig. 2). The oxygenator was an upright glass cylinder 60 cm high. Inflowing blood formed thin film on the inside of the glass and was oxygenated by mixture of oxygen and 5 % CO_2, introduced at the lower end of the cylinder. The blood was then transferred by roller pump into one of two glass perfusion reservoirs of 50 ml each. A valv was used to direct blood into one reservoir while the animal was perfused from the other and vice ra. The blood was forced into the animal by air from tw pressure tanks, where pressure could be varied independently. One reservoi was connected to the desired tank by solenoid valves, while the other was open to the tmosphere. Changing from one perfusion pressure to another thus only required the turning of tw handles. The blood re-entered the animal through a cannula in the descending thoracic aorta. The left common carotid artery and both brachial arteries were tied off to reduce the size of the ascular bed and thus to prolong the steady perfusion pressures. Blood pressure was measured in the right carotid artery. The riou steady pressures could be maintained for up to 3 min, and maximal perfusion time was 25 min. Nerve activity was usually recorded after 20—40 sec at each new pressure level and never after less than 3 sec. Recordings of left aortic nerv activity during perfusion of the aortic arch were obtained in 11 rabbits, and the results were grouped in 20 mm Hg intervals.

Having completed the recording of nerv activity the weights f the atria, the left entricle and septum, and the right entricle were determined after drying the parts with filter paper (Aars 1968).

Circular strips of 1.5 mm width were cut from the post-stenotic part of the ascending aorta (ASC) and aortic arch (ARC) and thei load-length relationships were investigated *in vitro* (Aars 1968 b). Basic length of the strips was measured after adaptation to 3 g load and subsequent elongations were calculated as per cent of the basic length. The loads resulting from stretching of the strips were measured by two wire strai gauges glued to the suspending beam and recorded on Leeds and Northrup potentiometric recorder. Application of Laplace's formula, T=PxR, allowed comparisons to be made between length of strips and calculated blood pressure. Circumferential elastic modulus was calculated as

$$E = \frac{\Delta g \times L \times 981}{\Delta L \times A} \text{ dyn/cm}^2$$

where Δg is the increase of load which produced ΔL increase of length (cm) from previous length L. A is the cross-sectional area of the strip and 981 the conversion factor from g t dyn. For comparison of elastic moduli between ASC nd ARC strips, the difference i thickness of strips in the two areas had to be accounted for. This was accomplished by relating all strip longations to a new corrected basic length where mean g/cm ere the same for all groups of strips (Aars 1968 b). Corrections were not made for reduction of cross-sectional rea of the strips with f rther stretch.

Strip longations were grouped in intervals of 8 % and comparisons of load-length lationships in the three groups of animals were made for each step. The thickness of the strips was assessed by the use of Vernier callipers or by weighi g.

The equipment and technique for i estigating aortic strips were not available before aortic nerv recordings had been completed in several animals, and only 7 aortic strips from eight 3D and nine 4W rabbits were therefore examined (T ble I). The results were compared to the previously reported data obtained with strips from seven normal rabbits (Aars 1968 b).

Results

Aortic dilatatio

After four weeks, the stenosis had resulted in dilatation of the pper part of the ascending aorta and the aortic arch in most nimal. The dilatation graduall receded in the first 1—2 cm of descending aorta. Fig 1 show the ngiogram from a 4W rabbit, where t the stenosis, aortic diameter h d been reduced by 48 % and the angiogram from a normal rabbit. The diameter tios in th s 4W rabbit were

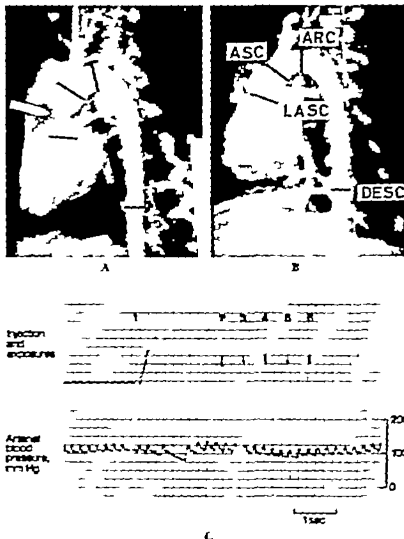


Fig 1
A. Aortogram from rabbit No 10 with aortic stenosis (arrow) of 4 week duration. Aortic diameters were measured as indicated by lines drawn on the films.
B. Aortogram from normal rabbit.
C. Recording of injection of contrast medium, of X-ray exposures (as given by numbers) and of carotid blood pressure during aortography in a normal rabbit.

aortic diameter ratios from the normal rabbit in brackets LASC DESC 1 49 (1.33) ASC DESC 1 (1 29) ARC DESC 1 43 (1 09) The 4W rabbit thus showed marked relative increases of upper ascending and aortic arch diameters and only a minor increase of the LASC DESC ratio Injection of the contrast medium caused some changes in heart rate and in blood pressure (Fig 1 C) The response to the injections varied from one animal to another This necessitated the use of one aortic dimension from each rabbit as its own control and reference value. The

TABLE I Aortic diameter ratios, performance of successful aortic nerve recordings, and basic lengths (Lo) of aortic strips in normal rabbits and rabbits 3 days (3D) and 4 weeks (4W) after production of ascending aortic stenosis.

	Aortogram ASC/DESC diameter ratio	ARC/DESC diameter ratio	Aortic nerve Recording Left	Right	Strip Lo mm ASC	ARC
3D rabbits						
1	1.39	1.36	+	+		
2		1.24	+	+		
3	1.58	1.40	+	+	17.0	15.5
4			+	+	18.5	18.2
5	1.66	1.41	0	0	18.2	15.7
6		1.56	+	0		17.4
7	1.71	1.57	+	+	18.6	
8	1.48	1.34	0	+	17.3	14.2
9	1.67	1.42	+	0	22.0	
10	1.63	1.44	+	+		19.4
	7	9	8	7	6	6
mean	1.59	1.44			18.6	16.7
S.E.	0.04	0.04			0.7	0.8
p[1]	<0.05	<0.001				< 0.10
4W rabbits						
1	1.63	1.27	+	0		
2	1.75	1.51	+	+		
3	1.43	1.38	+	+		
4	2.02	1.48	+	+		
5	1.45	1.46	+	+		19.4
6	2.09	1.53	+	+	23.0	16.8
7	1.36	1.42	+	+		14.2[2]
8	1.69	1.33	+	0	24.0	21.5
9		1.47	+	0		
10	1.77	1.43	+	0	23.5[2]	
11	1.93	1.64	+	0	20.7	17.0
12	1.73	1.51	+	+	22.2	16.0
13			+	0	22.7	19.0
14	1.81	1.49	+	+	20.3	15.7
n	12	13	14	8	7	8
mean	1.72	1.46			22.2	17.9
S.E.	0.07	0.03			0.6	0.8
p[1]	<0.001	<0.001			0.005	> 0.10
Normal rabbits						
	25	21	9	3	6	5
mean	1.48	1.29			18.6	17.3
S.E.	0.03	0.02			0.4	0.5

[1] Significance of difference between normal rabbits and rabbits with aortic stenosis, tested with Student T-test.

[2] Strip shortened during trimming procedures. Length not included in mean length of the

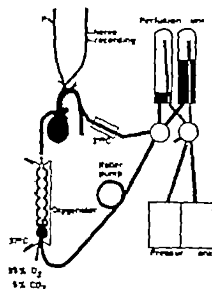


Fig. Schematic illustration of technique for perfusion of the aortic arch. Blood was drained from the right side of the heart int an oxygenator and pumped to the perfusion unit. From here the blood was alternately forced, by compressed air int the descending aorta from one of tw reservoirs while the other reservoir was being filled with blood f om the oxygenator

diameter 5 cm down the descending aorta was chosen as such a reference this being the diameter farthest removed from the tenosis that could be reliably measured on the same film as ascending aortic diameters.

Similar observations to those shown in Fig 1 were made in most of the other 4W bbits, except that the stenosis was found to ha e induced no significant pre stenotic dilatation of aorta (mean LASC/DESC ratio 4W 1 47 n = 13 normal 1 4 The degree and location of maximal post-stenotic dilatation varied between animals Table I Mean upper ascending aortic diameter was about 16 ς greater in 4W rabbits mean ASC/DESC ratio 4W 1 72, normal 1 48) and mean aortic arch diameter was about 13 greater than normal (mean ARC DESC ratio, 4W 1 46, normal 1 29

Both pre-stenotic mean LASC DESC ratio, 3D 1.52, normal 1 42, $p<0.05$) and post stenotic ascending aortic diameters (Table I m 3D rabbits were about 7 greater than normal, and mean aortic arch diameter was about 1? increased in the 3D animals mean ARC DESC ratio 3D 1 44 normal 1 29)

The animals lost weight the first few day after the thoracotomy and on the third day weighed on a erage 145 g less than before operation They rega ned weight, however and after 4 weeks had put on a mean 640 g Bod weights at the time of the nerve recording were not significantly different in the three groups of rabbits. The stenosis did not result n left entricular hypertrophy since mean left/right ventricular weight ratios were bout equal normal 3 4 3D 3 .. 4W 3 5)

Aortic nerve cordi g

The relationship between mean left aortic ers activity and diastolic pressure was

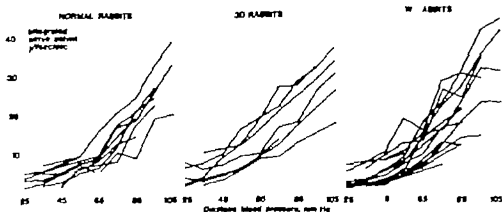


Fig. 3. Integrated left aortic nerve mean activities related to diastolic pressures in nine normal rabbits, nine rabbits with aortic stenosis of 3 days duration (3D) and fourteen rabbits with stenosis of 4 weeks duration (4W).

not significantly different in the three groups of rabbits, although variations from one animal to another were greater in the operated groups than in the control group (Fig. 3). Systolic activities were nearly similar in all three groups. As seen in Fig. 4 diastolic activity in 4W rabbits was higher than normal from 85 to 105 mm Hg (at 85 mm Hg: $p<0.02$, at 95 mm Hg: $p<0.005$ at 105 mm Hg: $0.10>p>0.05$) but at lower pressures, and for 3D animals at all pressures examined, the diastolic activities were not significantly different from those found in normal animals at comparable pressures.

Activity in the right aortic nerve seemed to fall into two groups. It was either high, similar to that found in left aortic nerves, or low showing only small increases with a rise of pressure (Fig. 5). Neither mean, systolic nor diastolic activities in the 3D and 4W rabbits deviated significantly from the pattern found in normal rabbits.

In perfusion experiments, the left aortic nerve activity adapted to new pressures in a few seconds, and remained practically unchanged over the following 20—40 sec (Fig. 6). The results from normal, 3D and 4W rabbits are plotted in Fig. 7. No consistent differences could be detected between rabbits from the three groups. As expected, the activity at steady pressures was somewhat higher than diastolic activity and lower than mean activity at pulsating pressures during intact circulation. The normal animal with lowest nerve activity at 90 mm Hg and the 3D animal with lower activity than the others from 110 mm Hg upwards, had both shown low activities during intact circulation.

Noise of the recordings was fairly constant amounting to 10—20 µV for the electrodes, or about 1 µV sec/sec when measured with the integrator, and was usually not taken into account. When, in a few cases, noise was high the difference between noise in these recordings and the usual noise in the other animals was subtracted from the integrated activities.

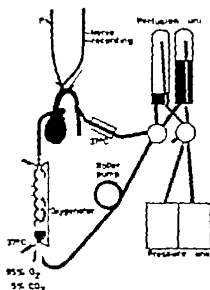


Fig. 2. Schematic illustration of technique for perfusion of the aortic arch. Blood was drained from the right side of the heart into an oxygenator and pumped t the perfusion unit. From here, the blood was alternately forced, by compressed air int the descending aorta from one of two reservoirs while the other eservoir was being filled with blood from the oxygenator

diameter 5 cm down the descending aorta was chosen as such a reference this being the diameter farthest removed from the stenosis that could be reliably measured on the same film as ascending aortic diameters.

Similar observations to those shown in Fig. 1 were made in most of the other 4W abbits except that the stenosis was found to ha e reduced no significant pre-stenotic dilatation of aorta (mean LASC/DESC ratio, 4W 1.47 n = 15 normal 1.4 The degree and location of maximal post-stenotic dilatation varied between animals Tabl 1 Mean upper ascending aortic diameter was about 16 % greater in 4W rabbits mean ASC DESC ratio 4W 1.72 normal 1.48) and mean aortic arch diameter wa about 13 greater than normal (mean ARC/DESC ratio, 4W 1.46, normal 1.29

Both pre-stenotic mean LASC DESC ratio, 3D 1.52, normal 1.42, $p<0.05$) and post-stenotic ascending aortic diameters Table 1) in 3D rabbits were about 7 greater than normal, and mean aortic arch diameter was about 12 increased in the 3D animals mean ARC/DESC ratio 3D 1.44 normal 1.29)

The animals lost weight the first few days after the thoracotomy and on the third day weighed on a erage 145 g less than before operation. They rega ned weight, however and after 4 weeks had put on a mean 640 g. Body weights at the time of the nerve recording were not significantly different in the three groups of rabbits. The stenosis did not result n left entricular hypertrophy since mean left/right ventricular weight ratios were bout equal normal 3.4 3D 3.2, 4W 3.5

Aortic nerve record ng

The relationship between mean left aortic nerv activity and dia tolic pressure was

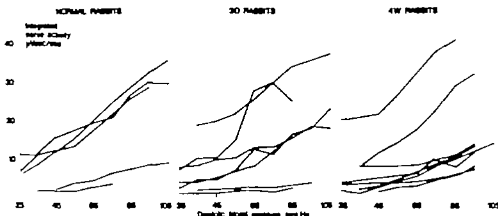


Fig. 5. Right aortic nerve mean activities in individual rabbits in the three groups of animals mentioned in Fig. 3.

relationships of ASC strips from operated and non-operated animals. As expected from Fig. 8, Young's modulus of elasticity was equal in the three groups of ASC and ARC strips. Calculation of load in terms of blood pressure showed that stretching ARC strips (Fig. 9) and ASC strips to 132 % of basic length corresponded to a rise in mean pressure from about 50 to 180—250 mm Hg. The slight differences in relative stretching of aortic wall at equal pressures in 4W and normal rabbits, shown in Fig 9 were due to the basic length being determined when the strips were loaded with 3 g The importance of Fig 9 is its demonstration of the fact that a given increment of pressure produced a roughly equal relative increase of aortic circumference in 4W and normal rabbits.

Unfortunately correlations could not be made between static load-length relationships of the aortic wall, as investigated *in vitro* and baroreceptor activity at steady perfusion pressures, because examination of strips was successfully carried out in only three of the eleven animals studied during aortic arch perfusion.

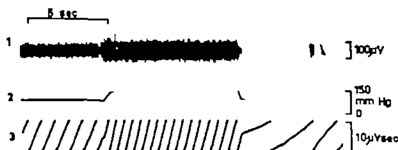


Fig. 6. Neurogram (1) and integrated activity (3) from left aortic nerve during perfusion of aortic arch in rabbit with aortic stenosis of 3 days duration. Blood pressure (2) measured in right common carotid artery

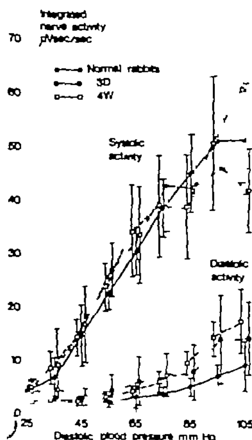


Fig 4 Average systolic and diastolic activities in left aortic nerv. In the same three groups of animals as in Fig. 3 The ertical line indicate ±2 S.E.

Mean heart rate calculated for each animal and based on the frequency at each recording of nervous activity was not significantly different in the three groups (normal 287 3D 300, 4W 290 beats/min) Initial blood pressures were also about equal (average alues. normal rabbits 129/99 3D 136/97 4W 141/96 mm Hg) but pulse pressures were highest in 3D and 4W animals ($p<0.05$)

Aortic strips

Strips from 3D and 4W rabbits were never found to vary in thickness from strips from normal animals, and the mean thicknesses of 0.36 mm and 0.26 mm found in ASC and ARC strips in normal rabbits (Aars 1968 b) were therefore used for all animals in the present series ASC strips from 4W rabbits were significantly longer than strips from normal and 3D rabbits, whereas ARC strips had about the same basic length with a 3 g load in all animals (Table I)

The load length relationship of ARC strips was nearly equal in all animals (Fig 8) ASC strips showed slightly less resistance to stretch than ARC strips at high elongations. No significant differences were found, however between load-length

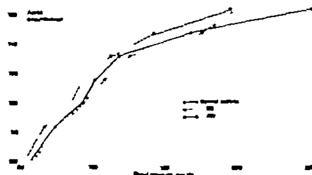


Fig 9 Approximate relationship between relative length of strips from aortic arch (aortic circumference) and arterial blood pressure. The formula T=P×R was applied to mean values of load and length in the three groups of strips.

diastolic) pressure and of pulse pressure (Gero and Gerova 1962) In Fig 3 and 4 left aortic nerve activity has only been related to diastolic pressure. Taking into account the increased pulse pressure found in 4W rabbits, systolic aortic nerve activity in these rabbits was in fact lower than would be expected in the left aortic nerve from normal animals with comparable blood pressures.

In 3D rabbits, the aortic arch was dilated to the same degree as in 4W rabbits, but the ascending aortic dilatation had not yet reached the 4W value. The forces causing maximal dilatation were apparently still at work, but even so, left aortic nerve activities were within normal limits.

Maximal dilatation was found in the upper part of the ascending aorta after four weeks, and most fibres from receptors in this area pass through the right aortic nerve. Influence of dilatation on pressor receptors should accordingly be most profitably studied in the right aortic nerve. Quantitative comparisons of activity in this nerve were not possible, however in all rabbits, as the right aortic nerve was thinner and more easily damaged than the left. The activity in the right aortic nerve varied considerably from one animal to another (Fig 5) but there was no indication that the dilatation had resulted in increased activity through this nerve. Most 4W rabbits actually had lower right aortic nerve activity than had 3D animals at *comparable* pressures.

The pressure-activity relationship in animals with dilated aorta was also normal at steady pressures. At all pressures studied, aortic baroreceptor activity in these rabbits was thus related to the blood pressure levels, and not to the degree of distension of the receptor area. This might be explained as a result of altered pressure-stretch relationship of the aortic wall, or by adaptation of the receptors. Only the first of these possibilities was examined, in that *in vitro* investigations of the static load-length relationship of the aortic wall were carried out. Acute elongation of aortic strips from normal rabbits to a length corresponding to the increased aortic diameter found in 4W rabbits resulted in a marked increase in the elastic modulus of the normal strips. At such a length the normal aortic strips showed greater resistance to further stretch than before (Fig 8). The strips from the 4W rabbits, which had reached this increased length (aortic circumference) gradually and over

four weeks, showed the same resistance to stretch as did normal strips at their normal length. In other words, the load length diagram of strips from dilated aortas in 4W rabbits was equal to that of the shorter normal strips. This observation is contrary to that made by Roach (1963 b) who found the post-stenotic segments of femoral and carotid arteries to be more distensible than normal in the area of maximal post stenotic dilatation. The discrepancy between the two investigations might be due to a different experimental approach. Roach studied the *in vitro* pressure-volume characteristics of canine arteries with non-functioning smooth muscles, whereas the present load-length studies were carried out in aortas where the smooth musculature was still functioning

The normal load-length relationship found in receptor area strips from the dilated aortas suggests that at comparable blood pressures the receptor elements of such aortic walls would be stretched to the same degree as would the receptor elements of normal aortic walls. This will explain why the pressure-activity relationship was similar at steady pressures in the two types of animals. It was thus unnecessary to postulate adaptation of receptors in steady state situations.

The dynamic behaviour of the aortic receptor area was not examined, and it is impossible to decide from the present study whether the discrepancy between ex pected and measured activity during intact circulation in rabbits with dilated aortic arch was due to alterations of the receptors or other wall elements. It was striking to find, however that, as in experimental hypertension (Aars 1968 a) the aortic baroreceptor activity had been reset Chronic increases in blood pressure or in diameter of the receptor area are accordingly of minor importance for aortic baroreceptor activity

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References

Aars, H Experimental post-stenotic dilatation of the ascending aorta in rabbits. *Act phy iol. sc d* 1963 *59* Suppl. 213 5
Aar H Aortic baroreceptor activity in normal nd hypertensive rabbits *A t physiol. sc d*, 1968 a. 7 298—309
Aar H Static load-length haracteristics of aortic strips from hypertensiv rabbits *Acta physiol sc d* 1968 b 73 101 —110
Aars, H and B Kristi sen A respirator for ot thoracic surgery in small animals *A I hir sc d* 1965 *129* 517 520
Aars, H and S Lin p A method for quantitative analysis of aortic nerv activity *J ppl Physiol.* 1968 *24* 416—418
Ba ewald, E W C Roberts A Goldb tt M M A cl S D Rock nd J W Gilbert Aortic stenosis Physiological, pathological and linical concepts *A n. nt M d.* 1963 *58* 494 5 2
Greo, J and M G Koy *The rol of parameters of pulsating pressure in the stimulation of intracarotid receptors. Arch nt Pharm ody* 1962 *140* 33—44
Roach M R., An experimental tudy of he production and time course of poststenotic dilatation in the femoral and arotid rteries of adul dogs *Circulat R* 1963 a. *13* 537 551
Roach M R. Changes in arterial disten i h a cause of poststenotic dilatation. *Amer J Cardiol.* 1963 b *12* 802—815
Robinson, S., K Ismo and M Movt An nal sis of mechanisms of post stenotic dilat tion. *A giology* 1967 *18* 349 36

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The Effect of Mannitol Sucrose, Raffinose and Dextran on Posthypertonic Hemolysis

By

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Abstract

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Inhibition of the hemolysis that occurs after first increasing and then decreasing the concentration of solutes in the surrounding medium of human red blood cells (posthypertonic hemolysis) was studied in *in vitro* experiments with regard t its possible importance for blood preservation by freezing procedures hemolysis after freezing and thawing is considered to be mainly due to such ariations in solute concentrations (Lovelock 1953) All the tested substances, *viz.* mannitol, sucrose, raffinose and dextran gave substantial decrease in hemoglobin (Hb) liberation. The effect of such substances when used as additives in studies of blood preserv tion by freezing may thus have found an additional explanation. The low molecular weight substances decreased Hb liberation mainly by reducing the number of hemolysed cells. Dextran, however decreased the amount of Hb liberated from the individual cell without an apparent decrease in the number of damaged cells. Thus when Hb liberation is decreased by the use of dextran, this reduction may not be good measure of the damage to the cell population. The consistency of the results with hypothesis for the mechanism of posthypertonic hemolysis is discussed. Assuming this hypothesis to be correct, the inhibitory effects of non-penetrating additives seem, t least partially t be also explicable.

Many substances, including sugars, glycols and polymers, inhibit the hemolysis of erythrocytes that occurs after freezing and thawing (Doebbler and Rinfret 1962) The mai cause of such hemolysis is assumed to be th rise in salt concentration occurring in connection with freezing and the reduction in this concentration connected with thawing (Lovelock 1953)

It has been suggested that the effect of additives d ring freeze preservation is d e to a reduction of the salt concentration rise d ring freezing (Lovelock 1954 Strumia, Colwell and Str mia 1960a) or t a reduction f the growth rat of ic crystals (Luyena and Rose 1956 Meryman 1957 Strumia t al 1960a) The possibil ty th t add ti es decrease th hemolysis that occurs after ertain hanges n salt concentration is largely untested. Experiments n which freeze hemolysis is reproduced only by ariations in concentrations of sol tes (posth per onic hemol without changes in temperature are relatively easy to perform, d n ol only a small

number of variables. Such experiments should be well suited for a close study of the hemolytic mechanism and the effect of additives on such systems, i.e. on condition that the additives act also directly upon posthypertonic hemolysis and not only in the way indicated above.

The aim of this investigation was to ascertain whether some substances which inhibit hemolysis connected with freeze-preservation will also inhibit posthypertonic hemolysis. The mechanism of such an inhibition is discussed on the basis of a hypothesis that posthypertonic hemolysis is an osmotic hemolysis, which is caused by the cells taking up solutes in the highly hypertonic medium, and after transfer to an isotonic or less hypertonic medium reacting in the same way as normal cells do in a hypotonic medium. This hypothesis of "paradoxical hypotonic hemolysis" was suggested by Söderström (1944) and discussed further by Zade-Oppen (1968).

The choice of additives for this investigation was restricted to a few substances which are usually denoted as nonpenetrants. By this is meant that normal erythrocytes have a relatively low permeability for these substances, in contrast, for example to glycerol.

Material and methods

Dextran [illegible]50 ($\bar{M}$ = [illegible]50,000, $\bar{M}$ = 125,000) was kindly supplied by Pharmacia, Uppsala, Sweden. All chemicals were of the highest grade of purity commercially available.

Blood was obtained by venesection from subjectively healthy human donors, immediately prior to the experiments. It was collected in centrifuge tubes containing heparin. The red cells were washed three times in 1[illegible] volumes of a solution containing 0.1[illegible]5 M NaCl, 0.005 M KCl and 0.025 M veronal-acetate buffer (Michaelis 1931) pH 7.40±0.03 (pH-meter model PHM 22b or model 26, Radiometer, Copenhagen, Denmark). This solution is henceforth called isotonic saline solution. After the last wash the cells were packed by centrifugation at 3000×g [illegible]0 min. A constant volume of cells (about 30 µl) was suspended in 0.[illegible] ml isotonic salt solution (= initial cell suspension). This also contained additives when such were used in all [illegible]es of the experiment. 1.00 ml hypertonic solution was quickly added. After 2 min [illegible]0 ml isotonic salt solution (containing additives when used) was added. From 0.5—2 hrs were allowed for completion of hemolysis before the samples were centrifuged and the supernatant analysed for liberated Hb. Centrifugation was performed either [illegible] 3000×g in [illegible] swing-out head or at 3 [illegible]00×g in an angle head. The tonicity was varied with NaCl. Solutions of dextran and salt were dialysed (Visking dialysis tubing, Union Carbide, Chicago, Ill.) against salt solutions during 3 days at +4°C in order to obtain the same chemical potential of electrolytes in the dextran solutions as in the corresponding salt solutions used for the control experiment (Laurent 1963). The degree of hemolysis was expressed as the fraction of Hb liberated from the cells in per cent. Hb was determined as the methemoglobin-cyanide derivative. To 4 ml of sample 0.1 ml of [illegible] reagent containing 8 g $NaNO_2$, 0.208 g KCN and 4 g $NaHCO_3$ per 100 ml was used (Zade-Oppen 1960). The total amount of Hb per sample was obtained from water hemolysates of red cells diluted parallel with the samples.

All experiments were performed at room temperature (22—25°C).

Further details are given by Zade-Oppen (1968).

For photomicrographic purposes experiments were performed with a slight modification. Packed cells were mixed directly with hypertonic solution, and after the desired time isotonic salt solution was added, the proportions being 1 : 40. Samples from cell suspensions obtained in this way were mounted between glass slides and cover slips and were sealed in with vaseline before photographing with phase contrast optics (Zeiss, West-Germany).

Results

The experimental conditions were chosen such as to obtain a strong but not complete hemolysis when additives were not used. The time chosen for the cells to be in contact with the hypertonic environment was, for practical reasons, 2 min. The salt

TABLE I. The effect of some additives on posthypertonic hemolysis.

The values give the fraction of Hb in the supernatant in per cent of the total Hb ± S.E. (n = 5). Experimental conditions: 2 min in 2.170 M, then 0.269 M saline. Additives present in all phases of the experiment (including the primary cell suspension).

Centrifugation was performed at 3000 × g for 10 min.

	With additive	Control
Dextran 250		
51 g/l	66.6 ± 1.0	72.3 ± 1.0
Raffinose		
0.005 M	76.0 ± 0.6*	
0.084 M	15.0 ± 0.2	
0.100 M	15.8 ± 0.4	82.3 ± 1.7
Sucrose		
0.100 M	19.8 ± 0.9	
Mannitol		
0.100 M	24.5 ± 1.5	

−4 −3

concentration (0.005 M KCl, 0.025 M buffer, xM NaCl) of the hypertonic cell suspension was therefore kept in the range 1.93 to 2.37 M (Zade-Oppen 1968).

Effect of dextran. Dextran inhibits posthypertonic Hb liberation (Table I and II). The values are somewhat misleading, however, since it was found impossible to obtain complete separation of cells and medium with the centrifugation procedure used (either 3000 or 37,500 × g) in the high dextran concentration present in the posthypertonic phase. On phase microscopy cells could always be seen in samples of supernatant containing such concentrations of dextran. The aim of the analysis of Hb in the supernatant was to obtain a value for Hb liberated into the medium. When the supernatant contained cells, however, the value obtained was thus erroneously high. The inhibitory effect of dextran in these cases was thus higher than is apparent from Table I and II. In one experiment (time of hypertonicity 2 min, hypertonic concentration 2.15 M) with dextran 44.2 g/l present in all phases of the experiment, a significantly (P>0.99) lower value for Hb liberation was obtained when centrifugation was performed at 37,500 × g instead of 3000 × g (5.0 per cent instead of 61.4 per cent). The amount of cellular Hb in the supernatant seemed to increase with dextran concentration. When this approached 100 g/l flotation of cells was observed. The effect of dextran was to reduce the liberation of Hb. This was significantly (P>0.99) lowered when dextran 51 g/l was present in all phases of the experiment (Table I). The measured inhibitory effect was, however, smaller than that of the low molecular weight carbohydrates. As the magnitude of the systematic error mentioned is unknown, this difference is somewhat uncertain.

TABLE II. The effect of dextran and raffinose on posthypertonic hemolysis when these additives were present during various phases of the experiment.

Additive in	Dextran 250 32.8 g/l A	36.5 g/l B	Raffinose 0.06 M C
hypertonic phase	35.3 ± 1 7	48.6 ± 0.7	73.2 ± 2.9
posthypertonic phase	27 6 ± 0.8	45.2 ± 2.9	20.9 ± 0 9
hyper and posthypertonic phases	32 1 ± 1.5	38.2 ± 0.9	24.8 ± 0.9
Control	37 6 ± 2.0	64 4 ± 0.7	58.7 ± 1 4

= 4

The values represent the fraction of Hb i the supernatant in per cent of the total Hb ± S.E. (= 5)

Experimental conditions

electrolyt molarity

Column	A	B	C
Hypertonic concentration	1 950	2.370	2.170
Posthypertonic concentration	0.255	0.280	0.269

The hypertonic phase had duration of 2 min.

Cen rifugation was performed t 3000 × g in columns A and C and t 37500 × g in column B; for 30 min in columns A nd B and for 20 mi In column C.

When dextran was used in the posthypertonic phase he subsequent centrifugation never resulted n complete separation of cells and medium The Hb in the supernatant is thus larger than the Hb actually liberated from the cells.

In two f rther experiments an attempt was made to ascertain in which phase of the experiment the dextran effect was exerted (Table II) In the one case the presence of dextran 32.8 g/l n the hypertonic phase alone gave no significant decrease in the degree of Hb liberation (centrifugation at 3000 × g) In the other case dextran 36.5 g/l. here also present in the hypertonic phase alone ga e significant decrease in Hb liberation (centrif gation at 37,500 × g) In both cases a gnificant ly lowered Hb liberation was obtained both wh n dextran was present in th post hypertonic phase alone and when it was present also n the hypertonic phase The results in columns A and B of Table II appear contradictory concerning the quest on of whether dextran used only in the hypertonic phase is in fact an inhibitor of Hb liberation. Such an inhibition seems to be possibl However it seems certa n that dextran used in the hypertonic phase only does not ncrease Hb liberation, as does raffinose

For reasons mentioned above the most reliable val for inhibition by dextran will probably be found in Table II column B (lower dextran concentration than in

T ble I best centrifugation) Dextran 36.5 g/l in hyper and posthypertonic phases reduced the Hb liberation by 41 per cent of the liberation in the control. When used in the posthypertonic phase alone the reduction was 30 per cent. Due to the systematic error the correct values for the inhibitory effect may be higher The order of magnitude found is about the same as obtained with similar concentrations of dextran in hypotonic hemolysis (Hjelm, Östling and Persson 1966, Davies *et al.* 1968) These results may not be quite comparable, however since the values obtained from the cited experiments on hypotonic hemolysis refer to the inhibition when Hb liberation without dextran was virtually complete.

Th effect of low molecular weight carbohydrates present d ring all phases. 0 1 M mannitol, sucrose or raffinose gave large decreases of Hb liberation (Table I) The effect of raffinose was significantly larger (P>0.99) than that of sucrose which in turn had a greater effect (P>0.99) than mannitol. 0.084 M and 0 1 M raffinose gave effects of the same order of magnitude, while 0.005 M ga e no inhibition.

I hibitio f hypert nic hemolysis. Since a minor part of the hemolysis studied is n hypertonic hemolysis (Zade-Oppen 1968) the effect of raffinose on such hemolysis was investigated. Red cells were exposed to 2.170 M salt solution for 3 hrs before centrifugation. Without raffinose a Hb liberation of 18.0 ± 0.9 (S.E., n=5) was obtained, and with 0 1 M raffinose $9\,7 \pm 0.8$ (S.E., n=5) per cent.

The eff ct f raffinose d ring the various phases of the experiment was investigated (Table II column C) When raffinose was present during the hypertonic phase alone, the resulting Hb liberation was *greater than in the control.* When this saccharide was present in both the hypertonic and posthypertonic phases, or in the posthypertonic phase alone, a marked inhibitory effect was observed. In the latter case a somewhat smaller (P>0.99) value for liberated Hb was obtained than when t was also present in the hypertonic phase. This is in agreement with the finding of a higher Hb liberation when raffinose was present in the hypertonic phase than when it was absent altogether (control)

The effect f varying affin se concent ations The inhibitory effect of raffinose was also studied under the same conditions as the experiments in Table I with varying raffinose concentrations (Fig 1 line a) It appears from the figure that a concentration of about 40 mM suffices to decrease the degree of hemoglobin liberation to a half. With increasing raffinose concentrations the slope of the curve becomes less steep and the inhibition is not complete t the highest concentration used, 0.160 M.

M ch ism f aff ose actio An experiment was performed in order to test the hypothesis that raffinose acts mainly by making th posthypertonic phase more hypertonic and thus red cing the difference in osmotic pressure between the cell

The standard error of the mean was calculated according to the formula S.E. $= \sqrt{\frac{\ldots -)}{(- 1)}}$

Significances were calculated according to Student t-test.

Fig. 1. Posthypertonic Hb liberation with varying conditions.

In one experiment (line a, squares) all samples were exposed to 2.170 M electrolyte ( ) concentration and then to 0.263 M e. In all phases of the experiment raffinose (Raff) was present in concentrations as shown on the abscissa. The inset (a) describes the experiment briefly, the ordinate representing concentration and the abscissa time.

In the other experiment (described by inset b) the concentration (only) was kept constant in initial cell suspensions and in hypertonic phases. In the posthypertonic phase as well as 0.269 M there was varying concentration ( ) of either Raff (dashed line b, open circles) or NaCl (dash-point line b, filled circles).

In all cases hemolysis decreased with increasing concentration in the final ("posthypertonic") phase.

(after this has taken up some solutes in the hypertonic phase) and its new medium. Both raffinose (Fig. 1, dashed line) and NaCl (Fig. 1, dash-point line) were used as "additives" in the last phase of the experiment. Hb liberation decreased with decreasing concentration differences between the hypertonic and "posthypertonic" phase. This liberation decrease was larger with raffinose than with NaCl. 0.0 M raffinose or an excess of 0.07 M NaCl reduced the Hb liberation to half the value obtained without an "additive". This difference would appear larger if the results were plotted on an osmolal scale instead of a molar one.

The resulting curve of the experiment where NaCl was used to vary the final concentration is in fact a so called "osmotic resistance (fragility) curve" for cells pretreated with a hypertonic saline solution. This was shifted a considerable distance into a higher concentration region in comparison with a normal osmotic resistance curve. The slope of this curve is however dissimilar as it crosses a wider concentration range than the normal curve.

Microscopic observation. The appearance of partially hemolysed cell suspensions obtained with the hemolytic systems reported in this paper were studied under phase contrast. The aim was to determine whether the hemolytic process of the individual cell proceeded to nearly total Hb loss as is the case during hypotonic hemolysis (Zade-Oppen and Marsden 1962) or whether it resulted in only a partial loss of Hb (as is the case when hypotonic Hb liberation is inhibited by macromolecules) (Marsden, Zade-Oppen and Johansson 1957; Davies, Marsden, Östling and Zade-Oppen 1968). Although the phase contrast microscope is not suited for quantitative measurements it can be used to differentiate between these two patterns of hemolysis: hemolysis where Hb liberation goes to near completion (all-or-none type) resulting

Fig. 2. The appearance in phase contrast of posthypertonically partially hemolysed cell suspensions.
a. With 0.08 M raffinose present in the posthypertonic phase only. Hypertonic solution 2.01 M. Duration of hypertonic phase 5 min.
b. With 0.08 M raffinose present in the hypertonic phase only. Hypertonic solution 2.57 M. Duration of hypertonic phase 5 min.
c. With 0.08 M raffinose present in the posthypertonic phase only. Hypertonic solution 2.57 M. Duration of hypertonic phase 5 min.
d. With dextran 36 g/l present in the posthypertonic phase only. Hypertonic solution 3.33 M. Duration of hypertonic phase 5 min.
Both when raffinose is present in the hypertonic phase alone (b) and in the posthypertonic phase alone ( ) tw distinctly different cell types re seen. Bright, seemingly unaltered cells (1) and faint "ghosts" (2). This indicates that the nature of hemolysis in the individual cell is of an all-or-none type. Occasionally cells which seemed to have lost only part of their Hb content also appeared when raffinose was used to inhibit hemolysis (3). A non-representativ high proportion of such ery dark cells surrounded by halo is seen in c.
When posthypertonic hemolysis was inhibited by dextran the pattern of hemolysis was different, almost all of the hemolysed cells having liberated only part of their Hb content, as judged from their high contrast (dark cells with halo). The same conditions but without dextran had yielded virtually complete Hb liberation.

in two easily distinguished types of cells (unaltered cells and faintly visible "ghosts") and the second type, where Hb liberation is incomplete (partial hemolysis) and the contrast of images of hemolysed cells varies with the amount of residual Hb (Davies Marsden, Östling and Zade-Oppen 1968).

Posthypertonic hemolysis in *NaCl systems* (without additives) is of the all-or none type (as also previously reported by Zade-Oppen 1968). When *raffinose* was used as an additive either during all phases of the experiment or during hypertonic or posthypertonic phases alone (Fig. 2, a and b) the appearance of the cells was that of the all-or none type. However cells w th a greater contrast than the common ghosts were occasionally seen (Fig ...). The impression was obtained that the number of such cells increased with increasing raffinose concentrations, but the observations were too few to be conclusiv. The supernatant after centrifugation at 5000 × g for 20 min of such a hemolysate with 80 mM raffinose was free from such cells w th an intermediate Hb liberation as well as from unhemolysed cells.

When *dextran* was used in the posthypertonic phase the microscopic picture was that of cells which had only liberated part of their Hb. Fig. 2, d (dextran 36 g/l) was obtained from a hemolytic system which without dextran would have resulted in nearly 100 per cent Hb liberation (3.53 M during 3 min) (Zade-Oppen 1968) 2 per cent of the Hb was recovered in the supernatant after centrifugation at $3000 \times g$ for 25 min. This supernatant, however contained very dark cells with a bright halo. This means that the average liberation of Hb into the medium must be less than 7? per cent. Nevertheless practically no unaltered cells were found in the hemolysate The refractive index of the medium was increased by the presence of dextran and hence the contrast was decreased in the phase contrast image. The quantitative effect of dextran 36 g/l (refractive increment $\alpha = 0.00151$) (Snyder Isbell, Dryden and Holt 1954) corresponds, however to a Hb concentration of only about ?8 g/l ($\alpha = 0.0019$4 (Stoddard and Adair 19?4) or less than 10 per cent of the Hb concentration of an average cell in a normal environment. It is therefore assumed that the effect of the presence of dextran on the contrast is of minor importance. It was thus concluded that posthypertonic hemolysis was altered by dextran from a pattern with a nearly all-or-none Hb liberation to one with a partial or none liberation.

Discussion

Inhibition of hemolysis reduced by freezing procedures and by increasing and then decreasing concentrations in the medium. The results obtained in this investigation ...ad demonstrate that the addition of mannitol, sucrose raffinose and dextra ... posthypertonic hemolysis as measured as a function of Hb liberation. The effect of nonpenetrating additives in freeze-preservation experiments may thus be due to a diminishing of the solute concentration increase in the medium during freezing Strumia, Colwell and Strumia 1960a, Rinfret 1963 and also due to b) the capability of the cells to better withstand the still existing variations in electrolyte concentrations during freezing and thawing. The latter factor may be of great importance as judged from the magnitude of inhibition obtained in experiments with posthypertonic hemolysis

Mechanism of posthypertonic hemolysis According to the hypothesis of paradoxical hypotonic hemolysis Soderstrom 1944 Zade-Oppen 1968) posthypertonic hemolysis is of an osmotic nature and its extent depends upon both the amount of solutes taken up by the red cells during the hypertonic phase of the experiment, and the osmolality of the final suspending medium As predicted from this hypothesis, hemolysis actually becomes smaller with increasing concentrations in the final medium (Fig 1 curves b

The experimental result can also be regarded as an osmotic resistance (or fragility) curve obtained with cells which were pretreated with a hypertonic solution. This resistance curve of pretreated cells had become displaced into a higher concentration region comparison with curves obtained with normal cells

This is to be expected when red cells contain a larger than normal amount of solutes (Wilbrandt 1941). The experimental result so far seems to offer strong support to the hypothesis.

However, there are two factors which may reduce the strength of this support, viz. 1) the altered slope of the resistance curve and 2) the obtained difference between the curve with NaCl concentration alone as variable and the curve in which the NaCl concentration was constant and the raffinose concentration was varied.

1. When the resistance curve is displaced into another concentration region this may be expected to occur without an alteration of the slope. The result obtained showed, however, a curve which covered a larger concentration range. The shape of the normal resistance curve reflects individual variations within the red cell population (Ponder 1948, p. 211). The obtained alteration of the slope may thus be explained as due to the pretreatment with strongly hypertonic media affecting the population in a non-uniform way. Similar displacements of the resistance curve into a region of higher concentrations with a decrease in slope have been reported after pretreatment with X-ray irradiation (Wilbrandt 1941), with incubation for 3 hrs in glucose-free bicarbonate buffer and with heating (Prankerd 1960). If this explanation of the altered slope is accepted, the results shown in Fig. 1 continue to give strong support to the hypothesis. It cannot be precluded, however, that the alteration of the slope is due to a combination of two different mechanisms of hemolysis.

2. When the final (posthypertonic) concentration was varied with NaCl, an increase in concentration (from 0.269 M) by about 0.07 M reduced Hb liberation by half. When the final concentration was varied by the use of 0.269 M electrolytes and xM raffinose, an increase of only 0.05 M was necessary for the same effect. According to the hypothesis the result should be mainly independent of the composition of the final medium and only dependent upon its osmolality. On an osmolal scale the difference found would be still larger due to the dissociation of NaCl. However, the osmotic action of a solution depends largely upon the membrane over which it acts. The red cell membrane is fairly likely to have a larger reflection coefficient (Staverman 1951) for raffinose than for Na and Cl ions. Whether the difference in reflection coefficient is large enough to make up for the difference in experimental results is, however, difficult to determine, since it was found that the hypothesis could be valid only if the cell permeability to solutes increased with increasing solute concentrations in the hypertonic medium (Zade-Oppen 1968). Therefore red cells pretreated with very hypertonic solutions may not be quite comparable with normal cells.

Summarizing the considerations under 1) and 2) above, the results obtained can still be taken as a support of the hypothesis, but the possibility cannot be excluded that two different mechanisms of hemolysis act simultaneously: (a) osmotic hemolysis resulting from the swelling of cells due to their total content of solutes (as according to the hypothesis) and (b) colloid-osmotic hemolysis of cation permeable cells which swell due to their content of Hb. This latter swelling should be counter-

acted by raffinose (Jacobs and Stewart 1947). In other words this means that it is difficult to differentiate between a situation in which all cells have a relatively much lower reflection coefficient for NaCl than for raffinose, and one in which part of the cells have a reflection coefficient for NaCl close to zero (cation permeable cells) and another part with higher reflection coefficients for NaCl. In either case a mechanism of hemolysis of the type assumed in the hypothesis (fully or for part of the cells) is possible.

Mechanism of inhibition of posthypertonic hemolysis when low molecular weight carbohydrates were used as additive during all phases of the experiment. As stated in the paragraph above it is understandable that less hemolysis is obtained if additives are present only in the final ("posthypertonic") phase of the experiment, making this more hypertonic. The same argumentation would hold also for the situation in which additives are present during all phases of the experiment, on condition that they do not influence or take part in the events during the first and second (hypertonic) phase. The primary influence to be considered in this connection is penetration of additive into the cell and an increase in membrane permeability to solutes in the hypertonic environment (Zade-Oppen 1968). When raffinose was used as additive in the hypertonic phase only the hemolysis was greater than in the control. This indicates that the cells may have taken up more solutes in the presence of raffinose. Whether this was due to an increase in NaCl permeation induced by raffinose or to the permeation of some raffinose into the cell, or a combination of both factors cannot, of course, be determined from the present experiments. The increase in uptake of solutes in the hypertonic phase must have been relatively small, however, in comparison to the effect of the same additive in the final (posthypertonic) phase, since raffinose when present in all phases reduced the hemolysis to almost as great an extent as raffinose present in the final phase only.

If the increased permeation of solutes into the cell comprises wholly or in part a permeation of the additive itself, it can be expected that an additive with a lower molecular weight will have a higher rate of permeation and will thus increase hemolysis to a greater extent when used in the hypertonic phase alone. If it is used during all phases of the experiment the inhibition of hemolysis caused by the additive will thus decrease with decreasing molecular weight. This was, in fact, the result obtained with mannitol, sucrose and raffinose (Table I). The same order in protecting effect obtained with these substances was found also in freeze thaw experiments (Doebbler and Rinfret 1962). It is difficult, however, to draw any conclusions from these results, as the substances used do not belong to a homologous series and their permeation through the cell membrane may also vary with factors other than molecular weight.

Effect of dextran on Hb liberation and cell damage. When dextran was used as an additive the Hb liberation was decreased. The effect cannot, however, be attributed to the same factors as the effect of the low molecular weight carbohydrates, since the dextran concentrations used were too low to have a significant osmotic effect. It was also found that dextran affects the pattern of posthypertonic

hemolysis in a similar way to its effect on the pattern of hemolysis in hypotonic media, i.e. hemolysis of the individual cell is not of the all-or-none type. Hb liberation is thus not a good indicator of the number of cells that have reacted to the lytic stimulus (Davies, Marsden, Östling and Zade-Oppen 1968) When dextran is used a decrease in Hb liberation therefore does not necessarily mean a decrease in the number of cells that ha e reacted. Cells which in the presence of dextran have reacted and have lost for instance a minor part of their Hb may well have lost a major part of other substances, mainly of a lower molecular weight than Hb, necessary to maintain the integrity of a functioning cell (Hjelm, Östling and Persson 1966) The pattern of hemolysis is altered by several macromolecules tested on hypotonic systems (Davies, Marsden, Östling and Zade-Oppen 1968) It is also likely that the effect observed on posthypertonic hemolysis is not specific for dextran.

The way by which dextran reduces the liberation of Hb from individual hemolysing cells is unknown and deserves further investigation.

It is thus concluded that macromolecules decrease the Hb liberation from a population of cells without decreasing, notably at least, the number of cells which are severely damaged by the variations in solute concentrations. Dextran and other macromolecules may therefore not be so suitable as additives in freeze-preservation as the low molecular weight nonpenetrants. This conclusion, drawn from experiments i vit is in excellent accord with results obtained by Strumia, Colwell and Strumia (1960 a and b) and Strumia and Strumia (1964) These authors found that dextrose, lactose, albumin and dextran, when used as additives in freeze thaw experiments, all greatly increased the recovery" of cells as estimated from the ratio of free to total Hb in the thawed preparation. The sugars also greatly improved the 24-hr survival in the circulation of cells frozen, stored, thawed and transfused. However the survival of cells was poor when albumin or dextran was used as additive.

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References

D VIES, H. G., N V B. MARSDEN & G. ÖSTLING and A M M ZADE-OPPEN The effect of some neutral macromolecules on the pattern of hypotonic hemolysis. *Act physiol. sca d.* 1968. 74 I press.

DOEBBLER, G. F and A. P RINFRET The influence of protectiv compounds and cooling and warming conditions on hemolysis of erythrocytes by freezing and thawing *Biochim biophys. Acta.* (Amst.) 1962. 58 449—458.

HJELM, M., & G ÖSTLING and A E G PERSSON. The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran. *Act physiol. scand* 1966 67 43—49

JACOBS, M. H. and D R. STEWART Osmotic properties of the erythrocyte XII Ionic and osmotic equilibria with complex externa solution *J G n Physiol.* 1947 30 79—103.

LAURENT T. C., The interaction between polysaccharides and other macromolecules. 5. The solubility of proteins in the presence of dextran. *Biochem. J.* 1963 *89* 253—257
LOVELOCK, J. E., The haemolysis of human red blood-cells by freezing and thawing. *Biochem. biophys. Acta* (Amst.) 1953 *10* 414—426.
LOVELOCK, J. E., The protective action of neutral solutes against haemolysis by freezing and thawing. *Biochem. J.* 1954 *56* 265—270.
LUSENA, C. V. and D. ROSE, Effect of rate of ice-crystal on hemolysis of erythrocytes. *Arch. Biochem.* 1956. *65.* 534—544
MARSDEN N. V. B., M. ZADE-OPPEN and L. P. JOHANSSON The effect of dextran on the dry mass distribution in osmotic hemolysis. *Exp. Cell Res.* 1957 *13* 177—181
MERYMAN, H. T. Physical limitations of the rapid freezing method. *Proc. roy. Soc. B* 1957 *147* 45 —459
MICHAELIS, L., Der Acetat-Veronal-Puffer. *Biochem. Z.* 1931 *234* 139—141
PONDER, E., *Hemolysis and Related Phenomena.* New York, Grune and Stratton 1948.
PRANKERD, T. A. J. Studies on the pathogenesis of haemolysis I. hereditary spherocytosis. *Quart. J. Med.* 1960. *29* 199—208.
RINFRET A. P. Some aspects of preservation of blood by rapid freeze-thaw procedures. *Fed. Proc.* 1963 *22* 91—101
SNYDER, C. F. H. S. ISBELL, M. R. DRYDEN and N. B. HOLT Optical rotations, refractive indices, and densities of dextran solutions. *J. Res. nat. Bur. Stand.* 1954 *53* 131—137
SÖDERSTRÖM N. Hemolysis by hypertonic solutions of neutral salts. *Acta physiol. scand.* 1944 *7* 56—68.
STAVERMAN, A. J. The theory of measurement of osmotic pressure. *Rec. Trav. chim. Pays-Bas* 1951 *70* 344—352.
STODDARD J. L. and G. S. ADAIR, The refractometric determination of hemoglobin. *J. biol. Chem.* 1923 *57* 437—454
STRUMIA, M. M. L. C. COLWELL and P. V. STRUMIA, The preservation of blood for transfusion. IV. In vitro recovery after freezing and thawing of red cells modified with sugars. *J. Lab. clin. Med.* 1960a. *56* 576—586
STRUMIA M. M. L. C. COLWELL and P. V. STRUMIA, The preservation of blood for transfusion. V. Posttransfusion survival of red cells modified with sugars, frozen and stored in the frozen state. *J. Lab. clin. Med.* 1960b. *56* 587—593
STRUMIA, M. M. and P. V. STRUMIA, Recovery and survival of human red cells frozen with albumin dextran and lactose-albumin. *In Proc. 9th Congr. Int. Soc. Blood Transf. Mexico 1962* ed. L. Hollinder Basel, New York, Karger 1964 pp. 61—68.
WILBRANDT W. Osmotische Natur sogenannter nicht-osmotischer Hämolysen. (Kolloidosmotische Hämolyse.) *Pflügers Arch. ges. Physiol.* 1941 *245* 22—52
ZADE-OPPEN, A. M. M. A method for the determination of hemoglobin in dilute solutions. *Acta Soc. Med. upsalien.* 1960 *65* 249—257
ZADE-OPPEN, A. M. M. Posthypertonic hemolysis in sodium chloride systems. *Acta physiol. scand.* 1968 *73* 341—364
ZADE-OPPEN A. M. M. and N. V. B. MARSDEN The phase contrast appearance of hemolysing red cells and the determination of fragility. *Acta Soc. Med. upsalien.* 1968. *73* In press

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Mechanical and Electrical Responses to Single Shocks in Developing Cat Leg Muscles Following Tetanization

By

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Abstract

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The effects of 9 sec tetanizations of varying frequencies on isometric twitch and electrical responses were studied in the gastrocnemius, soleus and extensor carpi radialis longus (ECRL) muscles of kittens of varying ages and adult cats. Nembutal® anaesthesia was used. In newborn kittens, no or only slight post-tetanic twitch potentiation (PTP) was recorded in the gastrocnemius muscle but a PTP of 80—90 % in the soleus. With time twitch potentiation increased in the gastrocnemius and decreased in the soleus. In the latter muscle of adult cats, PTP was obtained only after high-frequency tetanization (250—450 c/sec). This PTP proved to be due to repetitive activity and of different magnitude in Nembutal- and chloralose-anaesthetized animals. Changes in contraction and half relaxation time and in tension rise of the twitches occurred post-tetanically. At all ages, post-tetanic muscle action potentials, whether accompanying potentiated twitches or not, showed no consistent change in size, varying ± 10 % from the pre-tetanic values. In young kittens, post-tetanic potentials were slightly delayed. In the gastrocnemius and ECRL of adult cats, there was a short-lasting increase—as compared to the duration of twitch potentiation—in duration of the electrical response but no change in latency. It is concluded that, in Nembutal-anaesthetized animals repetitive firing accounts for PTP in the adult cat soleus muscle. The PTP in the soleus of young kittens and in the gastrocnemius and ECRL of the adult animals is, on the other hand, due either to a change within the contractile mechanism of the muscle fibres, or to a recruitment of muscle fibres.

Although post-tetanic potentiation (PTP) is a common finding in a wide variety of nervous paths, the cause of the phenomenon in various systems is not fully known (see review by Hughes 1958). In the neuromuscular system twitch potentiation might be brought about by (a) a change in the contractile mechanism of the muscle fibres, (b) repetitive discharge of some muscle fibres in response to single shocks post-tetanically and/or (c) recruitment of muscle fibres from a subliminal fringe. The results presented by Brown and von Euler (1938), Ramsey and Street (1941), Walker (1947), Bowman, Goldberg and Raper (1962), Standaert (1964) and Colomo and Rocchi (1965) favour the explanation given under (a). The results presented by Rosenblueth and Morison (1937), Feng, Li and Ting (1939) and also Standaert (1964) favour that given under (b). Many authors have tried to prove

the third possible explanation of twitch potentiation—recruitment of muscle fibres —but the compound muscle action potentials recorded during twitch potentiation have, in general, been found to be unchanged or decreased in size (see Hughes 1958)

The phenomenon of PTP of twitch tension has been found in all neuromuscular preparations studied in adult animals. However in 1965 Buller and Lewis, and Nyström and Skoglund, reported independently that, in kittens, there was a change with age in the capacity to produce post tetanic twitch potentiation. The results of Buller and Lewis (1965) also showed that, in young kittens, the future "fast-white and slow red" flexor hallucis longus (FHL) and soleus muscles, respectively produced about the same low magnitude of twitch potentiation, but around 5—6 weeks postnatally the two muscles were found to take different routes in their post tetanic behaviour In adult cats, not only the degree of PTP of twitch tension has been found to differ in "fast white" (FHL, tibialis anterior gastrocnemius) and slow red" (soleus) muscles, but also the mechanisms causing the PTP have been stated to differ in the two types of muscle (Standaert 1964) Nevertheless, the localization of the mechanism or mechanisms causing PTP of twitch tension is not fully understood (see Hughes 1958) Consequently the developmental changes observed by Buller and Lewis (1965) and Nyström and Skoglund (1965) raised the question whether changes in maturation of any particular part of the efferent path to muscle could be responsible for the developmental changes observed with regard to PTP of twitch tension. Before starting such an investigation, it was, however considered worth while to reinvestigate the developmental changes in PTP of twitch tension in "slow-red and fast white" muscle, since the reports of Buller and Lewis (1965) and Nyström and Skoglund (1965) were both of a preliminary nature. Thus in the present investigation, developmental changes in PTP of twitch tension were studied in the soleus, gastrocnemius and extensor carpi radialis longus (ECRL) muscles, and it was attempted to ascertain whether the cause of PTP is the same in kittens and adult cats.

Material and methods

Experiments were made on 42 kittens ranging in age from 3—140 days, and 5 adult cats The animals were anaesthetized with Nembutal® (40 mg/kg b w i p.) When experimenting on the hindlimb muscles—the gastrocnemius and soleus—the medial and lateral gastrocnemius nerves, the latter including the soleus nerve fibres, were exposed, and cut high up in the thigh. The remaining sciatic nerve was cut distally in the popliteal fossa as well as proximally in the thigh, and removed Similarly the ECRL nerve usually split into three branches, was freed from the main radial nerve for a long distance and cut, and the remaining radial nerve with its branches removed Care was taken not to destroy the blood supply to the muscles. Pools were formed in the popliteal and cubital fossae respectively and the nerves covered with paraffin oil at 37 C. The animals were kept warm by an adjustable radiator under the experimental stand and an infrared lamp, the rectal temperature being checked and maintained close to 37 C.

Stimulation was given by square-wave pulses of 0.2 msec duration Bipolar platinum electrodes were used. The stimulus intensity was well supramaximal for eliciting maximal twitch. In some preliminary experiments, the PTP of twitch tension was found to increase with duration of the conditioning tetanization up to 9—10 sec Since further increase in duration produced no major increase in PTP the conditioning tetanizations in the present investigation

were kept at constant duration, i.e. 9 sec. Four fixed frequencies were used. 45, 100, 250 and 450 c/sec, respectively their relative sequence being varied irregularly In the material presented, only those animals were included in which all or at least three of these frequencies were tested. Control as well as test shocks were delivered every 2.5 sec. The first test shock was delivered 2.5 sec after the end of the tetanization.

The animals were placed on conventional experimental stand. The hindlimb was fixed at hip and knee with drills, and t the ankle with clamp. The foreleg was fixed with drill through the distal end of the humerus, and with clamps at the shoulder and wrist.

The muscles used were freed from the surrounding muscles. The gastrocnemius and soleus tendons were detached along with part of the calcaneus bone, and connected to an isometric strain-gauge myograph through steel wire The ECRL tendon was sutured to the wire by loop. Muscle length was adjusted to give maximal twitches. During tetanization, the muscles contracted isometrically PTP could, however also be obtained when the muscles were completely slack. Between successive trials with different frequency tetanizations, the muscles were allowed to rest for 10—15 min.

Muscle action potentials were recorded by concentric needle electrodes with an outer diameter of 0.40 mm, the area of the active electrode being 0.1×0.5 mm. The potentials were fed to an AC-coupled amplifier system with high input impedance and an overall time constant of 200 msec, and displayed on an oscilloscope beam. By using some series of tetanizations, the position of the electrodes was adjusted so as not to move during or following repetitive stimulations Movement of the electrode was indicated by change in shape of the post-tetanic potentials as compared with the pre-tetanic ones. Muscle tension or time was displayed on the second oscilloscope beam. The oscilloscope screen was photographed on moving bromide paper

The various twitch characteristics were measured as given in Fig. 1 B and muscle action potential size was measured from baseline to peak. In 40 experimental trials, the muscle action potential size was measured both from baseline to peak and from peak to peak. The difference between these two ways of measuring with respect to the post-tetanic changes in muscle action potential size was not statistically significant on the 5 % level. In some experiments in adult cats, the area of the muscle action potentials was determined by planimetry

Results

Gastrocnemius muscle

Twitch tension Fig. 1 A illustrates the changes in twitch tension and size of muscle action potentials following a 9 sec tetanization at 45 c/sec in a 14-day-old kitten. As indicated in Fig. 1 B, twitch tension is measured to the peak (distance B—D) In the gastrocnemius of kittens of this age, little post tetanic twitch potentiation occurred. There were, however slight changes with respect to contraction time (distance A—B in Fig 1 B) and half relaxation time (distance B—C in Fig 1 B) as will be described in the following.

In Fig. 2 A—D the maximal PTP of twitch tension (filled circles) obtained in kittens of varying age, using four different frequencies and a fixed duration (9 sec) of the conditioning tetanization, is plotted against postnatal age in days. The open circles indicate the size of the muscle action potentials which accompany the maximal potentiated twitches. As seen, there was only slight or no PTP of twitch tension in the youngest kittens. Nor had still lower frequencies (15 c/sec) any effect in these kittens. With age, however there was a gradual increase in PTP and in the adult cat it usually amounted to more than 50 % of the pre tetanic control values

Although successive trials with one and the same frequency sometimes showed a certain variation as to the maximal amount of PTP of twitch tension produced, tendency was present for the lower two (45 and 100 c/sec) of the four different frequencies used to be more effective in eliciting twitch potentiation. This is ob-

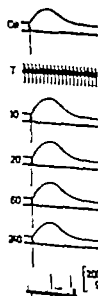


Fig 1 A shows records from an experiment on the gastrocnemius muscle of kitten 14 days old. The upper tracing in each record shows the twitch contraction curve and the lower tracing the accompanying muscle action potential. Co denotes the pre-tetanic control twitch. T denotes tetanization at 45 c/sec for 9 sec. The numerals indicate time in sec after cessation of the tetanization. Time marking every 10 and 100 msec

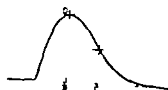


Fig 1 B shows the way by which the twitch characteristics were measured. Twitch tension = B—D Contraction time = A—B. Half relaxation time = B—C.

viously best seen when the PTP of twitch tension is high i.e. in old kittens and adult cats (Fig. 2 A—D)

The duration of twitch potentiation is illustrated in Fig 3 In kittens less than 30 days old, maximal potentiation was generally reached from 10—20 sec after cessation of the tetanization With increasing age maximal potentiation was reached sooner after the tetanization, and in the adult cat the first recorded twitch (2 5 sec after cessation of tetanization) usually showed the maximal potentiation (Fig 3) Although the values plotted in Fig 3 are those obtained in a single trial with a frequency of 45 c/sec the figure is representative of all frequencies used and of successive trials at the same frequency thus illustrating a general trend. In kittens, as well as in adult cats the PTP of twitch tension lasted for 6—10 min.

In Fig 2 E—H the changes in contraction time of the post-tetanic potentiated twitches, as compared to the pre tetanic ones, are plotted against postnatal age in days. After conditioning tetanizations of all four frequencies, the contraction time in young kittens was slightly lengthened This is also illustrated by the superimposed tracings in Fig 4 A. The lengthening can be seen during about 1 min post tetanically In kittens around 40—50 days old and older kittens as well as in adult cats little change took place in the contraction time of post tetanic potentiated twitches as

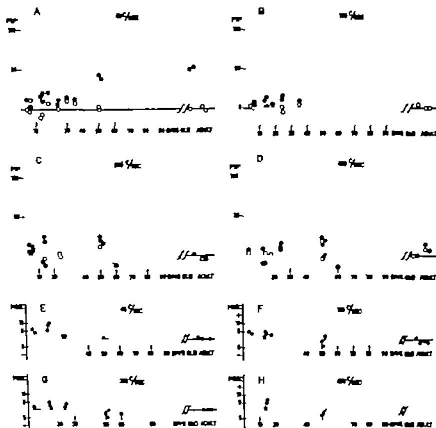


Fig. 2. I A—D maximal twitch potentiation obtained in the gastrocnemius muscle of each animal after 9 sec tetanization at the frequencies given in the figures is plotted (filled circles) against postnatal age. Twitch potentiation is expressed as percentage increase (or decrease) in tension over the tension of pre-tetanic control twitches. The change in size of the muscle action potentials accompanying the maximal potentiated twitches is plotted (open circles) in per cent of the size of pre-tetanic ones. I E—H the change in contraction time of the maximal potentiated twitches as compared with pre-tetanic ones is plotted against postnatal age. Increase in contraction time is marked + and decrease — See further text.

compared to pre tetanic ones (Fig 2 E—H Fig. 4 B—C) It should, however be noted that there was a steeper rise in twitch tension post tetanically If th rise in twitch tension had been equal in post and pre-tetanic twitches— in mph'bian muscle (Ritchie and Wilkie 1955)—a f irly marked ncrease should have occurred in the contraction time of post tetanic potentiated twitches compared to pre tetanic ones.

As seen in Fig 4 B and C a marked increase in half relaxation time of post a compared to pre tetanic twitches was recorded in ld k ttens and d lt cat Th is effect of a teta izat on was not as marked n young k ttens (Fig 4 A) 1 t lik twitch potentiation increased with age. In adult cat the ncrease half laxa



Fig 3. Duration of twitch potentiation in the gastrocnemius muscle of kittens 14 (circles) and 50 (squares) days old and adult cat (triangles) Twitch potentiation, expressed in % as given in Fig. 2, is plotted against time after cessation of 9 sec tetanizations at 45 c/sec.

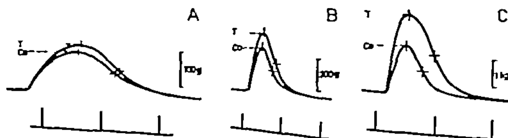


Fig 4 Superimposed tracings of single twitches of the gastrocnemius muscle before (Co) and after (T) 9 sec tetanizations at 45 c/sec in kittens 14 (A) and 50 (B) days old and in the adult cat (C) The post-tetanic twitch in A was induced 15 sec after cessation of the tetanization and those in B and C 2.5 sec after the end of the tetanization. The peak and the tension at half relaxation time are indicated. Time 100 msec See further text.

tion time following a tetanization was very pronounced, but only following low frequency (45 and 100 c/sec) tetanizations (see Fig 4 B and C) Using these frequencies, the maximal increase in half relaxation time was found to range from 8—11 msec in 5 adult cats and a 90-day-old kitten. The minimum increase in the same animals ranged from 4—10 msec. In view of the short contraction time of the gastrocnemius in adult cats—about 25 msec—these changes are conspicuous. When using high-frequency tetanizations (250 and 450 c/sec) however there was little change in the half relaxation time of post tetanic, potentiated twitches. In the 6 afore mentioned animals the range of change was from −3 to +4 msec The lengthening of half relaxation time decreased with time post tetanically as did twitch potentiation

Muscle action potentials As shown in Fig 2 A—D the muscle action potentials did not show the same post tetanic changes in size as the twitches. The action potentials plotted are those accompanying the largest potentiated twitches. As can be seen, action potentials slightly increased in size post tetanically occurred in kittens of all ages and also in adult cats, but potentials decreased in size post tetanically were also recorded. The changes in either direction did not exceed about 10 %. The post-tetanic changes in action potential size were apparently uninfluenced by the use of different frequencies in tetanization Fig 2 A—D

The time course of the small changes in action potential size varied in some experiments the potentials were restored to normal before recovery of the twitch, and

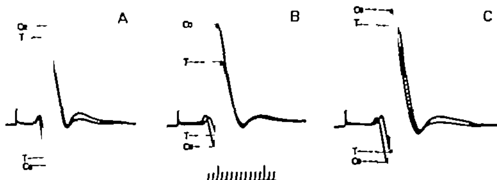


Fig. 5. Muscle action potentials recorded in the gastrocnemius muscle of kitten 3 days old before (Co) and 2.5 sec after (T) conditioning tetanizations of 9 sec duration at 15 (A) 45 (B) and 450 (C) c/sec. Time 1 and 10 msec. See further text.

in others they persisted changed throughout the period of twitch potentiation, and even longer. Repetitive potentials were never seen in the gastrocnemius, either in kittens or in adult cats.

In newborn kittens, little change was recorded in latency or duration of the muscle action potentials after a tetanization at 15 c/sec, as can be inferred from Fig. 5 A. In Fig. 5 Co denotes the pre tetanic control potential, and T the post tetanic test potential. In the same kitten as in Fig. 5 A, however an obvious increase in latency of the muscle action potential occurred after a 45 c/sec tetanization (Fig. 5 B) and after a 450 c/sec tetanization this change was still more pronounced (Fig. 5 C). This also applied to kittens around 14 days old (Fig. 6 A and B). These changes in latency of the muscle action potentials in young kittens persisted for 7—10 sec post tetanically.

In kittens around 50 days old, on the other hand there was not much change in latency (Fig. 6 C and D) but instead in duration of the muscle action potentials post-tetanically preferentially following a 45 c/sec tetanization (Fig. 6 C). Also in the adult cat, a change in duration of the potential without latency change occurred after a 45 c/sec tetanization (Fig. 6 E) whereas after a 450 c/sec tetanization there was no change in either (Fig. 6 F). The changes in duration of the muscle action potentials were most prominent during the first 15 sec following the tetanization, and after 30 sec the potentials were restored to normal (Fig. 6 E).

In some experiments in the adult cat, the area covered by the action potentials accompanying pre and post tetanic twitches was determined by planimetry. The maximal increase in action potential area post-tetanically was about 20 %. The duration of the change in area was fairly short, depending on the length of the change in duration of the potential. As seen in Fig. 6 E, the potential had returned to the pre-tetanic shape as soon as 30 sec after the end of tetanization. The increase in area of the muscle action potential might represent an increase in number of firing muscle fibres, recruitment of muscle fibres. However if this is the case

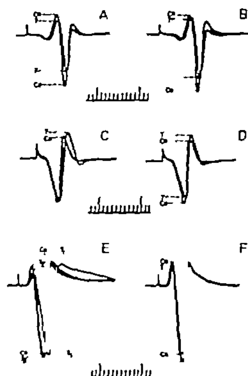


Fig 6

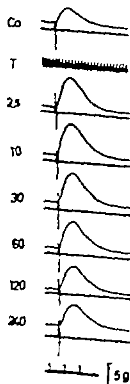


Fig. 7

Fi 6. Muscle action potentials recorded in the gastrocnemius muscle of kittens 14 (A—B) 50 C—D days old and of adult cat E—F) before (Co) and 2.5 (T T) or 30 (T) sec after the end of conditioning tetanizations at 45 (A, C and E) and 450 (B D and F) c/sec. Time 1 and 10 msec See f rther text

Fig 7 Records from an experiment on the soleus muscle of kitten 10 days old. The pper tracing in each record show the twitch contraction curve, and the lower tracing the ac companying muscle action potential Co denotes the pre-tetanic control twitch. T denotes tetanization at 45 sec for 9 sec The numerals indicat ime in sec after cessation of the tetanization Time marking every 100 msec

recruitment of muscl fibres should be a short-lasting phenomenon, which—as seen in Fig 3—could th be responsible for a tw tch potentiation of maximally 5—10 this being the magnit de of decrease in twitch tension during th first 30 sec post tetanically

Soleus muscle

Twitches Fig 7 illustrates the changes in tw tch tension and size of muscle action potentials in the soleus muscle of a 10-day-old kitten following a 9 sec tetanization at 45 c/sec. A considerabl twitch potentiation occurred (*cf* Fig 1) but no major change in size of the muscle action potentials post tetanically

Fig 8 A—D shows the maximal potentiation of tw tch tension obtained in kittens of varying age after tetanization for 9 sec t the frequencies given in the figure A

can be seen, a high PTP of soleus twitches occurred in young kittens. Great variations were, however observed. The maximal PTP declined with age, and in kittens older than 2 months little potentiation was noted. Although the lowest frequency used (45 c/sec) appeared to be most powerful in eliciting twitch potentiation, no major differences were present between the four frequencies (Fig. 8 A—D) In the adult cat, no PTP of twitch tension occurred after a low or moderate frequency tetanization (45 and 100 c/sec) In contrast, there was a slight decrease in twitch tension (Fig 8 A—B Fig. 9) at the post-tetanic time chosen for comparison, 10 sec (see the following) However after high-frequency tetanization (250—450 c/sec) the soleus muscles in some of the old kittens and adult cats showed twitch potentiation (Fig 8 C—D)

The time course of twitch potentiation is illustrated in Fig. 9 As seen in Fig. 9 A, maximal PTP of twitch tension was reached after 15 sec in the 4-day-old kitten, but earlier in the 45-day-old one, as was also found in the gastrocnemius (cf Fig. 3) No PTP occurred in the 140-day-old kitten. In contrast, old kittens and adult cats often showed a slight decrease in twitch tension during the first 15—20 sec after cessation of the tetanization. Since at all other ages both gastrocnemius and soleus muscles showed a maximal or nearly maximal post tetanic effect around 10 sec after cessation of a tetanization, this time was chosen for comparison in the adult cat soleus muscle. As seen in Fig 9 A, recovery to the pre tetanic level occurred later The twitch potentiation seen in the kitten soleus muscle lasted for 6—10 min, as in the gastrocnemius muscle of the adult cat. The PTP seen in the soleus of the adult cat after high-frequency stimulation (Fig. 8 C and D) differed definitely in nature from both the PTP in the soleus of kittens and that seen in the gastrocnemius of adult cats. It reached maximal potentiation after about 15—20 sec, and at 2 min after the end of the tetanization there was no longer any potentiation (Fig 9 B)

Fig. 8 E—H shows the difference between the contraction time of post and pre-tetanic twitches in the soleus at various ages. As can be seen, there is no consistent change post-tetanically in contraction time of the potentiated twitches obtained in the soleus of young kittens. At this age, no difference was present between the effect of the various frequencies used n tetanization (Fig 8 E—H) In older kittens and adult cats, on the other hand, low frequency tetanizations (45 and 100 c/sec) were followed by twitches with decreased contraction times (Fig 8 E—F) After high-frequency tetanizations (250 and 450 c/sec) twitches with fairly unchanged contraction times were observed but also twitches with greatly increased contraction times (Fig. 8 G—H) In Fig. 10, superimposed tracings from experiments on the soleus muscle of two k ttens, 4 and 140 days old are shown to illustrate the changes in contraction time noted post-tetanically It can be pointed out that where twitch potentiation was highest—i.e in the soleu of oung k ttens and in the gastrocnemius of adult cats—both muscles showed post-tetanic tw tches with f rly nchanged contraction times (cf Fig 10 A and 4 C)

However with respect to post-t tanı changes in half relaxation time, potentiated twitches in the young kitten soleus and the ad ıl t gastrocnemius differed. In the

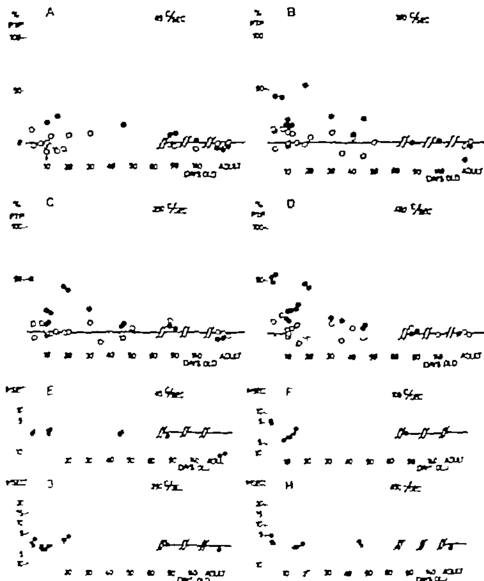
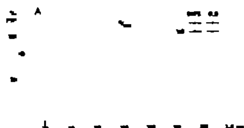


Fig. 8. Maximal twitch potentiation obtained in the soleus muscle of each animal (filled circles) after 9 sec tetanizations at the frequencies given in the figures is plotted, as in Fig. 2, against postnatal age in A–D. The change in size of accompanying muscle action potentials in % of pre-tetanic ones is indicated by the open circles. In E—H the change in contraction time of post- as compared to pre-tetanic twitches is plotted (cf. Fig.). See further text.

gastrocnemius of adult cats a consistent increase in half relaxation time occurred post-tetanically and the increase was marked in relation to the fairly short contraction time (see above). In the soleus of young kittens on the other hand, no consistent change in half relaxation time took place post-tetanically. In about half of the specimens, an increase in half relaxation time was noted, being of about the



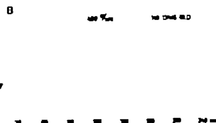


Fig. 9 Graph A shows the time course of twitch potentiation in the soleus muscle of kittens 4 (circles), 45 (squares) and 140 (triangles) days old. Twitch potentiation, expressed in per cent as given in Fig. 2, is plotted against time after cessation of 9 sec tetanization at 45 c/sec. Graph B shows the time course of twitch potentiation in the soleus of the kitten 140 days old after 9 sec tetanization at 450 c/sec. Cf Figs. 8 D 9 A and text.

same absolute magnitude as in adult cat gastrocnemius muscle, but the change was not as marked, in view of the long contraction time of the soleus in young kittens (see Buller Eccles and Eccles 1960 Buller and Lewis 1965) However in about half of the cases, no change in half relaxation time occurred post-tetanically in young kitten soleus muscle. An example is shown in Fig 10 A.

Following a low frequency tetanization (45 and 100 c/sec) in the soleus of adult cats and older kittens—where twitch potentiation is absent—there was a decrease in half relaxation time post tetanically as in contraction time (Fig. 10 B) Following high-frequency tetanizations (250—450 c/sec) however twitches with clearly increased half relaxation time occurred, these twitches also showing an increased contraction time (Fig 10 C)

Electrical recording Similar twitches to those in Fig 10 C are also illustrated in



Fig. 10. Superimposed tracings of single twitches of the soleus muscle before (Co) and after (T) 9 sec tetanizations at 45 c/sec in kittens 4 (A) and 140 (B) days old C is the same type of illustration in the 140-day-old kitten, but following 9 sec tetanization at 450 c/sec after which repetitive activity occurs. The post-tetanic twitches in A, B and C were induced 15 2.5 and 10 sec after cessation of the respective tetanizations. Note contraction and half relaxation time of pre- and post-tetanic twitches Time 100 msec. See further text

Fig 11 A, where they are seen in the electrical recording to be accompanied by a second deflexion after the main potential. The second deflexion usually appeared about 15 msec after the main potential, and was observed only after high-frequency tetanization in the soleus muscle of old kittens and adult cats. Moreover it was fairly small in the Nembutal-anaesthetized animals (Fig 11 A) whereas in those anaesthetized with chloralose it was extremely large, and the concomitant twitch showed a much higher potentiation (Fig. 11 B) than that in the former animals (*cf* Fig 11 A) The second deflexion on the electrical recording also appeared about 15 msec after the main potential in the chloralose-anaesthetized animals (Fig 11 B) After a small dose of eserine, greatly increased twitches were recorded without tetanization, and some small deflexions on the electrical recording were seen concurrently (Fig 11 C) After tetanization, the twitches recorded in the eserine treated specimens were greatly intensified, and were accompanied by a distinct repetitive discharge (Fig 11 C) Here as well, the second potential appeared about 15 msec after the main one (Fig. 11 C) Consequently the deflexions seen post-tetanically in the electrical recordings from muscles of chloralose and Nembutal-anaesthetized animals also probably reflect a repetitive discharge from some muscle fibres.

A repetitive discharge was never observed in the soleus of old kittens and adult cats after low frequency tetanizations, nor in the soleus of young kittens after any of the frequencies tested. The single muscle action potentials recorded at maximal twitch potentiation showed no consistent change in size from the pre tetanic level (Fig 8 A—D) The variation in size amounted to about ±10 % around the pre tetanic value

In the soleus of young kittens, the changes in latency of the muscle action po- ... recorded post-tetanically were similar to those in the gastrocnemius (*cf* Fig ... and 6 In adult cats, on the other hand, there was no increase in duration of the muscle action potentials post-tetanically in the soleus following a low frequency tetanization (45 c/sec) contrary to the gastrocnemius.

Ext... ...a ...radialis longus mu cle

As seen in Fig 12 A–D this muscle in kittens about 2 weeks old and younger showed a PTP of twitch tension amounting to some 40—50 % of the pre-tetanic twitch values, as compared to 10—20 % in the gastrocnemius muscle (*cf* Fig ? A—D) In the adult c t t th PTP was higher in the ECRL muscle than that in the gastrocnemius.

In kittens, the amount of PTP induced appeared to be about the same after all four frequencies used whereas in adult cats low frequency produced higher PTP At all ages the time course of the twitch potentiation was similar to that noted in the gastrocnemius (/ Fig 3

As can be inferred from Fig 12 E—H the changes in contraction time of post tetanic twitches of ECRL, as compared to pre-tetanic ones, were also similar to those in the gastrocnemius / Fig 2 E—H) In adult cats, however there seemed to be a more definite tendency in the ECRL than in the gastrocnemius for

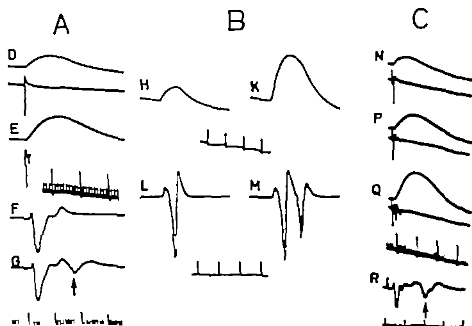


Fig. 11 Records obtained from experiments on the soleus muscle of adult cats anaesthetized with Nembutal (A) chloralose 80 mg/kg (B) and Nembutal with later i injection of eserine (C) Upper tracing in D, E, N P and Q shows the twitch contraction curve, and lower tracing the muscle action potential.

D is pre-tetanic control, and E is obtained 20 sec after the end of 9 sec tetanization at 450 c/sec. An increase in twitch tension and in contraction and half relaxation time occurs post-tetanically and second deflexion on the electrical recording (E) appears about 15 msec after the main potential, as seen in G (arrow) from another experiment. F is control and G is obtained 10 sec after the end of 9 sec tetanization t 250 c/sec Time for D—E 10 and 100 msec, for F—G 1 and 10 msec

In the chloralose-anaesthetized cat (B) K is obtained 22 5 sec after the end of 9 sec tetanization at 450 c/sec. H is the control. Note high twitch potentiation (cf D—E) and large repetitive potential—see M (25 sec after the tetanization) L is the control Time in H—K 100 msec in L—M 10 msec

In the eserinized cat (C) N is control before eserinization, P is obtained 10 min af er i injection of eserine (0.2 mg/kg) and Q 60 sec after the end of a 9 sec etanization at 100 c/sec in the eserinized cat. A repetitive discharge is already indicated in P and in Q tw potential waves are learly seen and third is indicated. As seen in R, the second potential (arrow) appears about 15 msec after the main one Time in N P and Q 10 and 100 msec, in R 1 and 10 msec. See further text.

post-tetanic potentiated tw tches to exhibit an increase n contraction time. This was probably due to the higher twitch potentiation in th ECRL The post tetanic changes in PTP and contraction time are shown Fig 13 The lengthening of contraction time was most conspicuous shortly afte, th tetanization, as was twitch potentiation, nd gradual recovery occurred w th time

As in the gastrocnemius and soleus m scles there was no increase in amplitud of the muscle action potentials accompany ng potentiated twitches at any age (Fig

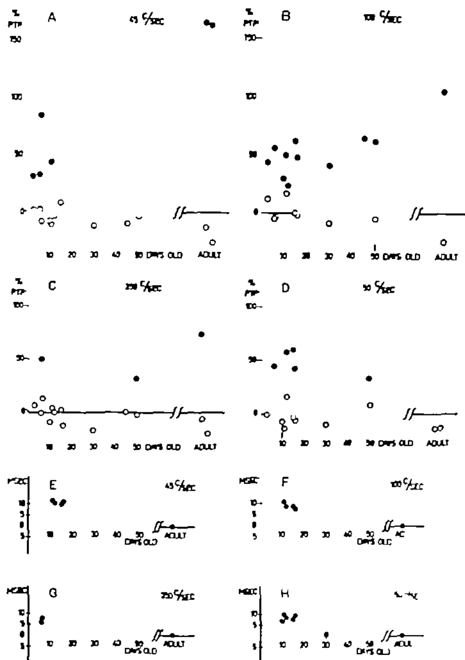


Fig. 12. Maximal potentiation obtained in the ECRL muscle of each animal (filled circles) after 9 sec tetanizations at the frequencies given in the figure is plotted, as in Fig. 2, against postnatal age in A—D. The change in size of the accompanying muscle potentials in per cent of pre-tetanic ones, is indicated by the open circles. In E—H the change in contraction time of post as compared to pre-tetanic twitches is plotted as in Fig. 2. See further text.

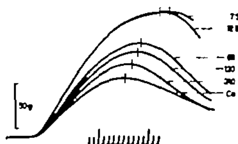


Fig. 13 Superimposed tracings of single twitches before (Co) and at various times after (as given by the figures in sec) the end of 9 sec tetanization at 100 c/sec ECRL muscle of kitten 60 days old. Note changes in contraction time. Time markings indicate 1 and 10 msec. See further text.

12 A—D) At all ages, any changes in latency and duration of the muscle action potentials after a conditioning tetanization were similar to those in the gastrocnemius (*cf* Fig 5—6)

Discussion

From the results presented, it seems reasonable to concl de that the PTP recorded in the adult cat soleus muscle after high-frequency tetanization is due to repetitive firing of some muscle fibres, as previously found by Feng, Li and Ting (1939) in decerebrate cats and by Bowman *et al.* (1962) and Standaert (1964) in chloralose anaesthetized cats. The potentiation in the soleus reported by Standaert (1964) is much higher than that found here in Nembutal-anaesthetized animals. This dif ference can probably be explained by the difference in anaesthesia (see Werner and Kuperman 1963) which is well illustrated by Fig 11 A and B. The consistent failure to record a repetitive discharge in the gastrocnemius and ECRL muscles at all ages, as well as in the soleus muscle of kittens, simultaneously with a PTP of twitch ten sion suggests that the mechanism of twitch potentiation is another in these cases than in the soleus of adult cats. This is further substantiated by the observations on frequency dependence and time course of twitch potentiation. In Nembutal-anaesthetized animals, the repetitive discharge appeared in the adult cat soleus after high-frequency tetanization only whereas twitch potentiation in the soleus of kittens, and especially in the gastrocnemius and ECRL, was highest after low frequency tetanizations. Furthermore, in the latter muscles, the PTP of twitch tension lasted for 6—10 minutes, whereas that due to repetitive discharge had a maximum duration of only about 2 minutes (Fig 9) Apparently therefore, twitch potentiation in the gastrocnemius and ECRL in Nembutal-anaesthetized animals of all ages and in the soleus of young kittens must be due either to a change n the contractile mechanism of the muscle fibres, or to recruitment of muscle fibres from a subliminal fringe of fibres.

As already pointed out in Results the short-lasting increase n area and duration of the muscle action potentials post-tetanically in the adult cat gastrocnemius and ECRL muscles might, actually indicat recruitment of muscle fibres contributing to twitch potentiation, although only to 5—10 % When no post-tetanic ncrease in

area or duration of the muscle action potentials occurs, the consistent lack of increase in amplitude of the potentials appears to disprove the possibility of a recruitment of muscle fibres. However as pointed out by several authors (Brown and von Euler 1938, Brown and Harvey 1938, Bernhard von Euler and Skoglund 1941 Walker 1948) temporal dispersion of the individual fibre potentials, or a partial muscle fibre depolarization due to accumulation of external potassium ions following the tetanization (Fenn 1939) might conceal an increase in number of firing fibres.

If then, recruitment of muscle fibres from a subliminal fringe were to be the basis of PTP of twitch tension, and the mechanism which counteracts the expected increase in size of the muscle action potentials in the adult cat is also present in the gastrocnemius of kittens, one would expect the post tetanic muscle action potentials to be decreased in this case since the kitten gastrocnemius muscle has no PTP No consistent decrease in size of the action potentials post tetanically was, however recorded in this muscle in kittens.

If activation of muscle fibres from a subliminal fringe is the basis of PTP of twitch tension in both the soleus of young kittens and the gastrocnemius of old kittens and adult cats, the results obtained here would indicate a disappearance of the fringe of muscle fibres with age in the soleus, and appearance of a fringe in the gastrocnemius. As regards the latter muscle this would imply a lack of a fringe of muscle fibres in the youngest kittens, or—if a subliminal fringe is present—an inability to activate muscle fibres from this fringe.

If a subliminal fringe is lacking all muscle fibres are fired by the pre-tetanic shocks. This does not seem unlikely since muscle fibres in young rats have been found highly excitable (Diamond and Miledi 1962, see also Katz and Thesleff 1957) In this case, however the results concerning PTP would indicate a difference between the excitability of the soleus and the gastrocnemius muscle fibres in young kittens.

The other possibility—an inability to activate muscle fibres from an existing subliminal fringe—would be analogous to the conditions with respect to PTP in the monosynaptic path of young kittens (Skoglund 1960 1966) Skoglund (1960) found that the absence of PTP in the monosynaptic reflex path in young kittens could be explained by hyperpolarization of the muscle afferents to block during repetitive stimulation Thus, even if a subliminal fringe were to exist additional motoneurons would not be activated (Skoglund 1966) As the post tetanic delay in muscle action potential in young kittens shows (Fig 5—6) a slowing of conduction velocity occurs, probably due to certain degree of hyperpolarization of the nerve terminals (*cf* Lloyd 1949 Wall and Johnson 1958 Skoglund 1960)

Skoglund (1960) found the monosynaptic response to be completely absent for about 6 sec following a 1 sec tetanization at 470 c/sec in newborn kittens. In the present investigation, however the size of post tetanic twitches in the gastrocnemius of young kittens was not found to be decreased. For this reason, the small latency increase recorded post tetanically in kittens—probably caused by nerve hyperpolarization—was not investigated in detail

If the lack of twitch potentiation in the young kitten gastrocnemius were to be due to hyperpolarization of some nerve terminals to block during tetanization, the appearance of PTP in the soleus of equally young kittens would indicate a difference in maturity of the respective nerve terminals, provided that recruitment of muscle fibres is the basis of PTP in both muscles.

From the results presented, recruitment of muscle fibres cannot be excluded as at least a partial explanation of twitch potentiation. The post-tetanic effects on twitch tension and muscle action potential size can, however also be explained by changes in the contractile system within the muscle fibres. If this is the case, muscle fibres in the soleus and gastrocnemius apparently differ in young kittens, as well as in adult cats, as regards their capacity to increase tension following a tetanus.

The possibility of different mechanisms for twitch potentiation in the soleus and gastrocnemius obviously also exists. In this connexion, it can be pointed out that, although kitten soleus and adult cat gastrocnemius show about equal magnitude of twitch potentiation, measured in % of the pre-tetanic twitch tension, a difference is present between them with regard to changes in half relaxation time (Figs. 4 B and C, 10 A) as well as in the post-tetanic changes in duration of the muscle action potentials.

The increase in duration and area of the muscle action potentials recorded post tetanically in the gastrocnemius and ECRL of adult cats might be due to a real increase in number of firing fibres and/or desynchronization of the individual muscle fibre potentials. If desynchronization exists, it is uncertain whether it is brought about by an appearance post tetanically of a larger range of conduction velocities in the nerve trunk, a change in the junctional transmission, or a change within the individual muscle fibres. Action potentials increased in d ration post tetanically were also observed by Walker (1948) in dult rat gastrocnemius muscle. Here, the change lasted for about 3 min. Colomo and Rocchi (1965) on th other hand, recorded action potentials increased in duration in a single amphibian nerve muscle fibre preparation where desynchronization obviously cannot exist.

If recruitment of muscle fibres is the cause of PTP the post tetanic changes in contraction and half relaxation time observed here might also be due to desynchronization, but the underlying cause might equally well be some change in the contractile system within the muscle fibres. Bowman *et al* (1962) found the contraction time of post tetanic twitches in the tibialis anterior muscle of adult cats to be increased, and suggested this and twitch potentiation to be due to prolongation of the active state of muscle contraction (Hill 1949 1953) This was found by Richie and Wilkie (1955) to be the cause of twitch potentiation in amphibian muscle. In single amphibian muscle fibres. Ramsey and Street (1941) observed prolongation of twitch relaxation with or without prolongation also of contraction time post-tetanically whereas Colomo and Rocchi (1965) found the contraction time of potentiated twitches to be decreased

The steeper rise in tension of post as compared to pre-tetanic tw tches found here (Fig. 4 10, 13) was also recorded in the cat tibialis anterior by Bowman *et al.*

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Post Tetanic Decurarization in Developing Cat Leg Muscles

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Abstract

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The effect of conditioning tetanization on the twitch tension and size of compound muscle action potentials of subseque t twitches has been studied in partly curarized gastrocnemius and soleus muscles of kittens, ranging in age from 3—140 days, and adult cats. Nembutal® anesthesia was used. Comparisons were made with result obtained in non-curarized animals.

At all ages, post tetanic increase in twitch tension in both muscles, when partly curarized, was accompanied by similar increase in size of the muscle action potentials.

I adult cats, high-frequency tetanizations (250—450 c/sec) were more fIecth than low (45 c/sec) in eliciting the decurarizing ffect. I young kittens, both were about equally effectn

The effect of tetanization on the half relaxation time of post-tetanic twitches was found to differ in normal and partly curarized gastrocnemius muscles, whereas this did not apply to the soleus of young kittens.

I both muscles, the post-tetanic effects seen in non-curarized and in partly curarized muscles differed with respect to the muscle action potential hanges, as w ll as to the time course and frequency-dependence of twitch potentiation. This indicates that the PTP of twitch ension found normal muscles is, most probably brought about mainly by a mechanism other than recruitment of muscle fibres

It is generally agreed that post tetanic potentiation (PTP) of the twitch tension and end plate potential occurs in the neuromuscular system. The compound muscle action potentials ha e however been found by most workers to become smaller than or remain about equal in ize to the pre tetanic ones during the stage of twitch potentiation (see review by Hughes 1958) Recruitment of muscle fibres as the underlying cause of twitch potentiation has therefore been doubted However in the rat gastrocnemius (Walker 1948) and cat gastrocnemius and extensor carpi radialis longus (ECRL muscles (Nyström 1968 a) of adult animals a slight increase in duration of the muscle action potentials has been noted post tetanically This change in duration of th muscle action potentials nevertheless lasted for a much shorter period than that during which a twitch potentiation took place

The capacity for twitch potentiation has been shown to change in the cat d ring postnatal development Buller and Lewis 1965 Nyström and Skoglund 1965) In

the gastrocnemius of newborn kittens, the post tetanic twitch potentiation (PTP) has been found to be low increasing gradually with age whereas in the soleus a high PTP was recorded in young kittens, its magnitude decreasing with age (Nyström 1968 a) The compound muscle action potentials recorded in both muscles after conditioning tetanizations—irrespective of whether the potentials accompanied highly potentiated twitches or fairly unchanged ones—were found, at all ages, to be of about the same size as the pre tetanic potentials, except in the adult cat gastrocnemius, in which a brief increase in duration of the potentials occurred post tetanically (Nyström 1968 a)

No explanation could be given of the difference between the PTP of twitch tension in the two muscles of both kittens and adult cats. Since the localization of the mechanism causing PTP of twitch tension is also unknown (see Hughes 1958) studies were made of various parts of the efferent path to the two muscles, i.e. of the nerve fibres (Nyström 1968 b) motor nerve terminals (Nyström 1968 c) subneural pparatuses and the cholinesterase (ChE) activity of the end-plates (Nyström 1968 d) Although the results obtained showed that various differences exist between the two muscles with respect to these parameters they were unable to further the understanding either of the mechanism of PTP or of the nature of the underlying differences between the two muscles with regard to PTP However in sections, the soleus end-plates were found to have a lower acetylcholinesterase (AcChE) activity than gastrocnemius ones, which might explain the readiness for repetitive activity in the soleus (Feng Li and Ting 1939 Standaert 1964 Nyström 1968 a)

Thus, the question remains whether or not the PTP found at any age in the two muscles is due to recruitment of muscle fibres from a subliminal fringe. The possibility of an appearance and disappearance in the gastrocnemius and soleus, respectively of a subliminal fringe of muscle fibres with age has been discussed previously in connexion with the postnatal ncrease in number of nerve fibres (Nyström 1968 b)

It is known that a subliminal fringe of muscle fibres exists in partly curarized muscles and that, following a tetanization, recruitment of fibres from the fringe occurs in these preparations (see Brown and von Euler 1938, H tter 1952, Liley and North 1953) Partly curarized animals of varying ages were therefore used in the present nvestigation, in order to compare the effects obtained post tetanically in these muscles where recruitment of muscle fibres occurs with those previously observed in normal muscles (Nyström 1968 a)

Material and methods

Experiments were made on 35 kittens ranging in age from 3—140 day and on 4 adult cats. The animals were anaesthetized with Nembutal® (40 mg/kg b w i p) The gastrocnemius and soleus muscles were used. The experimental arr ngements were he same as those described in preceding paper (Nyström 1968) As in hat tudy fixed duration of the conditioning tetanizations (9 sec) and four fixed frequencies were used 45 100 250 and 450 c/sec, respectively Control and test shocks were deli red every 2 5 sec. The first test shock was given 2.5 sec after the end of the tet zation

Curare (d-Tubocurarine Abbott) was i jected intraveneously through catheter in the left external jugular ein. I kittens, curare diluted in saline, 1 10, was used. The animals were artificially entilated.

Muscle action potentials were recorded with concentric needle electrodes, as previously described (Nyström 1968 a) Muscle action potential size was measured from baseline to peak (f Nyström 1968) as was twitch tension. Measurements of the muscle action potential area were considered unnecessary since the hanges observed in amplitude of the potentials were fairly large. The degree of increase in size of post-tetanic twitches and muscle action potentials is expressed as percentage of the pre-tetanic values. Since the degree of curarization differed from animal to animal, no comparisons are made between animals with respect to the maximal degree (in per cent) of potentiation (=decurarization) Comparisons will be made only within animals t fairly constant level of curarization. This level, which is given in the legends to each figure was always maintained to give partial neuromuscula block only

Results

Fig 1 shows the effect of a conditioning tetanization on the twitch tension and size of the muscle action potentials of subsequent twitches in the soleus muscle of a 6-day-old kitten, before (Fig 1 A) and after (Fig 1 B) partial curarization. Before curarization, there was considerable post tetanic potentiation (PTP) of the twitch tension, but the accompanying muscle action potentials were of about the same size as before the tetanization (Fig. 1 A) as reported elsewhere (Nyström 1968 a) In the partly curarized state, the same muscle also showed potentiation (decurarization) of the twitch tension, but this effect was accompanied by a similar change in size of the muscle action potentials (Fig 1 B) A further difference between the two post tetanic phenomena is seen in the duration of the twitch potentiation In the non-

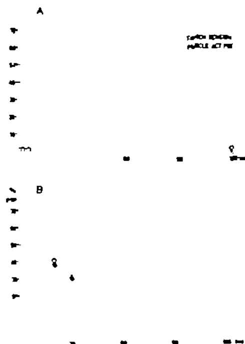


Fig. 1 6-day-old kitten anesthetized with Nembutal I A, the post-tetanic potentiation of twitch tension (filled circles) after a 45 sec tetanic stimulation of 9 sec duration is shown for the soleus muscle No concomitant increase occurs in size of the muscle action potentials (open circles) In B the same muscle is partly curarized, twitch tension being 65 % of the pre-curare les l. Here a 9 sec tetanic stimulation at 45 c/sec is followed by n increase in both tw tch tension and size of the muscle ac ion potentials. Post tetanic increase in twitch and action potential size is plotted in % of the pre-tetanic alues (ordinate) gainst time in sec after cessation of the cond tioning tetanization abscissa)

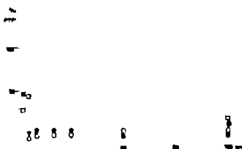


Fig. 2. 30-day-old kitten anesthetized with Nembutal. Soleus muscle I the non-curarized state, little or no post-tetanic change occurs in size of twitch (filled circles) and muscle action potential (open circles) I the same muscle when partly curarized (twitch tension 51 % of pre-curare level) an equal tetanization induces an increase in size of both twitch (filled squares) and muscle action potential (open squares) Tetanization frequency 100 c/sec, 9 sec duration. Plotting as in Fig. 1

curarized muscle, about half of th maximal potentiation obtained was still present 2 min after the end of the tetanic stimulation, whereas in the partly curarized muscle no effect persisted at that time. The time to maximal effect was, however similar in both preparations.

With age, the ability of the normal soleus muscle to show PTP of twitch tension declines (Buller and Lewis 1965 Nyström 1968 a) Fig 2 shows an experiment in a 30-day-old kitten where, in the non-curarized state, no post tetanic increase in twitch tension or size of the muscle action potential occurred. The decurarizing ef fect of a tetanization of equal duration and frequency was, however well shown by the same muscle when partly curarized (Fig. 2) Similar results were obtained in adult cat soleus muscle.

In non-curarized young kittens, there is little or no PTP of the twitch tension in the gastrocnemius muscle (Buller and Lewis 1965 Nyström and Skoglund 1965 Nyström 1968 a) If partly curarized, on the other hand, a considerable post tetanic increase in both twitch tension and size of the muscle action potentials is obtained This is illustrated in Fig 3 A—D where A shows the effect of a 9 sec tetanization at 45 c/sec in the normal non-curarized gastrocnemius muscle of a 7-day-old kitten. The PTP of the twitch tension amounted to some 10 % (n reality 10 g) of its pre tetanic level, but no change in size of the muscle action potentials occurred. When the same muscle was partly curarized (Fig 3 B) and subjected to a conditioning tetanic stimulation of equal duration and frequency to that in Fig 3 A, the post tetanic twitches showed a maximal potentiation (decurarization) of around 50 % (70 g) with a similar increase in size of th ccompanying muscle action potentials. When the frequency of the tetanic stimulation was increased to 100 /sec (Fig. 3 C) with the same duration (9 sec) the PTP of tw tch tension (decurarization) was more pronounced and amou ted t some 80 % (5 g) f the pre tetanic control alue. The muscle ction potentials w re also ncreased in size A further increase in frequency f the conditioning tetanization appeared t ha little additional effect on twitch tension (Fig 3 D) The action pote tials were however relatively more increased in size which ill trates that th rear n size of the muscle action potentials need not nec ssarily be of th same m gnitude (per cent) a the increase in twitch t nsion This is d to th experimental situation the action po-

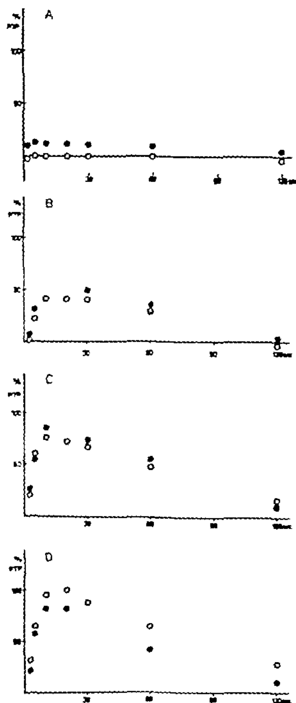


Fig. 3. 7-day-old kitten. Nembutal anesthesia. Gastrocnemius muscle. Plotting as in Fig. 1. Filled circles indicate twitch tension and open circles size of the muscle action potentials. The post-tetanic changes in twitch tension and size of the muscle action potentials in the non-curarized muscle are seen in A. Tetanization for 9 sec at 45 c/sec. In B, C and D the same muscle is partly curarized (twitch tension being 40, 34 and 50 % respectively of the pre-curare level). All tetanizations were of 9 sec duration. Frequency of tetanizations 45 c/sec in B, 100 c/sec in C and 250 c/sec in D. See further text.

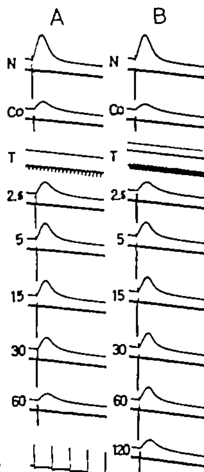


Fig. 4 25-day-old kitten anesthetized with Nembutal. In each record the muscle twitch is seen in the upper tracing, and the muscle action potential in the lower. N denotes normal non-curarized muscle, Co pretetanic level after partial curarization and T tetanization of 9 sec duration at the frequencies 45 c/sec (Fig 4 A) and 450 c/sec (Fig 4 B) The numerals on the following records indicate time after cessation of tetanic stimulation. Time markings every 100 msec.

tentials are recorded with a concentric needle electrode which records potentials from only a fraction of the muscl mass, whereas the twitch tension recorded is that of the whole muscle.

As seen in Fig. 3 the maximal decurarizing effect was chieved 10—15 sec after the end of the conditioning tetanization. irrespectively of the frequency of the latter Similarly the duration of the effect appeared similar after all three frequencies Fig 3 B—D)

In kittens more than 2 weeks old and in dult cats the duration of the effects of conditioning tetanizations of low and high frequencies clearly differed. This is illustrated I Fig 4 A—B nd in Fig 5 for the gastrocnemius muscle of a 25-day-old kitten. The decurarizing effects of tetanizations of 45 and 450 /sec on both twitch tension and size of muscle action potentials are seen in Fig 4 A and B, and the time course of the decurarizing effect on twitch tension Fig 5 Following a con-

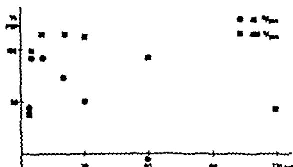


Fig 5. The post-tetanic increase in twitch tension seen in Fig. 4 after equally long tetanizations at 45 c/sec (circles) and 450 c/sec (squares) is plotted against time after cessation of the tetanization. The decurarizing effect is seen to increase with increasing frequency 25-day-old kitten. Gastrocnemius muscle Nembutal anesthesia.

ditioning tetanic stimulation of 9 sec duration with a frequency of 45 c/sec the decurarizing effect ceased within about 1 min, whereas after an equally long tetanization with a frequency of 450 c/sec, a considerable effect persisted even 2 min after cessation of the tetanization (Fig 5) Following a low frequency tetanization (45 c/sec) the decurarizing effect was at its maximum 5—10 sec after the end of the tetanic stimulation, whereas following a high frequency tetanization (250—450 c/sec) the maximal effect was obtained later i.e. around 15—20 sec after cessation of the tetanic stimulation

With increasing age the duration of the decurarizing effect of a low frequency tetanization (45 c/sec) decreased, and that of a high-frequency one (250—450 c/sec) increased (cf Figs. 1 B 3 B—D and 5) These effects were similar in the soleus and gastrocnemius muscles.

In the adult cat the non-curarized gastrocnemius muscle shows a high PTP of twitch tension (Standaert 1964 Buller and Lewis 1965, Nyström 1968 a) but not of the accompanying muscle action potentials (Nyström 1968 a) This is illustrated in Fig 6 A. However in the partly curarized gastrocnemius muscle of the adult cat a post tetanic effect was seen on both twitch tension and size of the action potentials (Fig 6 B) Furthermore the time course of the effect differed distinctly in the two preparations In the non-curarized preparation, maximal PTP of twitch tension appeared as soon as 2.5 sec after the end of the tetanic stimulation, and even after 2 min about half of the maximal PTP obtained persisted (Fig 6 A) The decurarizing effect on the other hand, did not reach its maximum until about 20 sec after cessation of the tetanic stimulation, and at 2 min the effect had almost subsided (Fig 6 B) Twitches obtained during the period of decurarization were never found to be larger than those before curarization.

In non-curarized normal gastrocnemius muscle there is little change in the contraction time of potentiated post tetanic twitches when compared to pre tetanic ones, but a marked increase takes place in half relaxation time (Nyström 1968 a) This is illustrated in a 25-day-old kitten in Fig 7 A where N is the pre tetanic normal twitch and T the post tetanic one When this muscle was partly curarized (C) a slight lengthening of half relaxation time occurred but no major change in contraction time as compared to the normal twitch before curarization (N) About

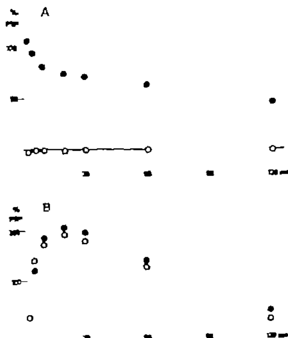


Fig 6. In A, the post-tetanic potentiation of twitch tension (filled circles) as seen in a normal non-curarized gastrocnemius muscle of adult cat, is plotted against time after cessation of 9 sec tetanization at 100 c/sec. There is no change in size of the muscle action potentials (open circles) B shows the effect of an equal tetanization in a partly curarized (twitch tension being 16 % of pre-curare level) gastrocnemius muscle of another adult cat. During decurarization, the muscle action potentials are also increased. A further difference between normal post tetanic potentiation and decurarization is seen in the time course and duration of the effects. Nembutal anesthesia.

the same contraction time was shown by post tetanic twitches (T) in the partly curarized muscle following an indirect tetanization for 9 sec at 45 c/sec. If these potentiated twitches, obtained during the period of decurarization, showed any change in half relaxation time, there was a decrease (T) as compared to the pre-tetanic twitch (C) Thus, tetanization of the normal muscle induced an increase in half relaxation time and no major change in contraction time (*f* N and T) whereas tetanization of partly curarized muscle induced no major change in either of these times, and if a change in half relaxation time did occur it was in the form of a decrease (*cf* C and T_2)

The soleus of young kittens showed about equally large PTP of twitch tension (measured in per cent of the pre tetanic control twitch) as the gastrocnemius of adult cats. In both preparations, the contraction time of potentiated twitches was about the same as that of pre tetanic ones. However the two preparations differ with respect to changes in half relaxation time (Ny from 1968 a) Fig 7 B shows superimposed tracings from an experiment on the soleus muscle of an 11-day-old kitten. As seen, there was little difference between the half relaxation time of pre (N) and post tetanic (T) twitches (*cf* Fig 7 A) The contraction time of the two twitches was also fairly equal Also when the muscle was partly curarized (C) the contraction time remained fairly constant whereas the half relaxation time reased slightly Following a 9 sec tetanization at 45 /sec in the partly curarized muscle, post-tetanic twitches (T) showed no change in contractio time and light de-

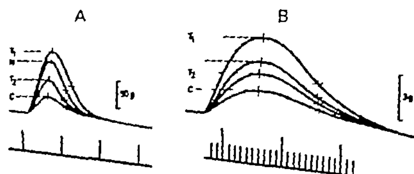


Fig. 7 A shows superimposed tracings from an experiment on the gastrocnemius muscle of a 25-day-old kitten. N is the control twitch in the normal, non-curarised animal, and T_1 twitch appearing 7.5 sec after cessation of a 9 sec tetanization t 45 c/sec. Post-tetanically there is no change in contraction time but marked increase in half relaxation time. C is the control twitch after partial curarization, and T_2 a twitch ppearing 15 sec after cessation of a 9 sec tetanization at 45 c/sec Here also contraction time is unchanged half relaxation time does not increase but, in contrast, decreases slightly (cf C-T_2) B is the same type of illustration but for the soleus muscle of an 11-day-old kitten. As in the gastrocnemius (f Fig. 7 A) all twitches show about the same contraction time There is no hange in half relaxation time post-tetanically in normal muscle (N T) nor in the same muscle when partly curarized (C-T_2) T_1 ppeared 20 sec and T_2 12.5 sec after cessation of 9 sec tetanizations t 45 c/sec. Time in A 100 msec in B 10 and 100 msec See further text.

crease in half relaxation time Thus in the soleus of young kittens, the effect of a tetanization on contraction and half relaxation time is fairly equal in normal (cf and T_1 and partl curarized muscle (cf C and T_2)

Discussion

In the gastrocnemius of the adult rat (Walker 1948) and cat (Nyström 1968 a) a brief increase in d ration of the muscle action potentials occurs post tetanically Most authors ha e however failed to observe any consistent change in amplitude of the muscl action potentials during the period of twitch potentiation, but recruit ment of fibres ha not been denied (see Hughes 1958) Various authors have suggested that temporal dispersion of the individual muscle fibre potential or partial muscle fibre depolarization produced by the tetanus might obscure a real increase in number of firing f bres as might also electrode movements during tetanization (see Hughes 1958

If however recru tment of muscle fibres really takes place in normal muscles, electrode movement an hardly explain the general failure of these muscles to show muscle action potentials increased in amplitude post tetanically This is be cause in the same muscles when partl curarized and with the lectrodes left in th same position, decurarization recruitment of fibres Liley and North 1953) is consistently accompanied by action potentials increased in ampl tude (Fig. 1)

Temporal dispersion might on th other hand occur and be caused by changes in the nerve at the neuromuscular junction and or in the muscl fibres Tetanization is

known to cause hyperpolarization of the nerve fibres (Lloyd 1949) and, in view of *fibre size, there might well be a differential decrease in conduction velocity of these* fibres (Wall and Johnson 1958, Skoglund 1960 a) Hyperpolarization is however likely to occur in curarized preparations as well (Liley and North 1953) but the desynchronization which should follow obviously does not prevent the muscle action potential from increasing Thus hyperpolarization of the nerve fibres as the cause of a desynchronization does not seem to be an explanation of the lacking increase in size of the muscle action potentials following tetanization of normal muscle.

Post-tetanically the end plate potentials are increased in size (Hutter 1952, Liley and North 1953 Gage and Hubbard 1966) which might hasten the transmission at any individual junction. It is not, however known whether or not any change takes place in the synchronization of the potentials post tetanically

A desynchronization due to differential changes in the individual muscle fibres following tetanization might occur Colomo and Rocchi (1965) demonstrated a decrease in conduction velocity post tetanically in single amphibian muscle fibres. This possible influence of a tetanization on the muscle fibres is likely to differ in normal and in partly curarized muscle, since in the former the muscle fibres are maximally contracted during tetanization, whereas in the latter they are not (Feng et al. 1938)

In *partly curarized muscles, the time course of the change in size of the muscle* action potentials post-tetanically is similar to that of the change in twitch tension (= decurarization) In normal muscle, on the other hand, there is either no change in size of the muscle action potentials, or a brief slight increase in duration, much briefer than that of twitch potentiation (Nyström 1968 a) Apparently therefore, recruitment of fibres takes place in curarized muscle until twitch tension reaches the normal, pre-curarization level. Another mechanism is probably responsible for a further increase in tension. In the gastrocnemius, this supposition is further strengthened by the findings with respect to half relaxation time of post and pre-tetanic twitches in normal and in partly curarized muscles (Fig 7 A) This does not, however apply to the soleus (Fig 7 B)

Besides the differences between non-curarized and partly curarized muscles with respect to the post tetanic changes in muscle action potential size, differences also exist as regards the time course and frequency dependence of twitch potentiation. In normal gastrocnemius muscle low-frequency tetanization has a more powerful effect in causing twitch potentiation than high frequency tetanization (Brown and von Euler 1938 Nyström 1968 a) whereas in partly curarized muscle the situation is the reverse (Fig. 5) In non-curarized soleus muscle of the adult cat, PTP is obtained only after high-frequency tetanization. The time course of this PTP (*cf* Nyström 1968 a) is similar to that in partly curarized muscle (Fig 2) The PTP in the normal soleus was found to be caused by repetitive discharge (Feng, Li and Ting 1939 Standaert 1964 Nyström 1968 a) whereas decurarization (= PTP in partly curarized muscle) denotes recruitment of fibres Both phenomena are probably based on the increase in end plate potential size that occurs post-tetanically (Hutter 1952,

Liley and North 1953, Gage and Hubbard 1966). The higher tendency of the soleus than of the gastrocnemius to show repetitive discharges is probably to be ascribed to the lower cholinesterase activity of soleus end-plates (Nyström 1968 d).

There seems little doubt that the decurarization phenomenon is due to a facilitating effect of the tetanic stimulation on transmitter release (Liley and North 1953). Since decurarization is found not only in soleus and gastrocnemius muscles of the adult cat but also in young kittens, this facilitating effect of a tetanization on the nerve terminals must be functioning already in kittens a few days old. Since, furthermore, decurarization in these young kittens is well shown after a high-frequency tetanization (250—450 c/sec), some of the potentials apparently reach the terminals, and induce the facilitated state of transmission. This, in turn, implies that the failure of the non-curarized gastrocnemius muscle in young kittens to show PTP of twitch tension (see Nyström 1968 a) cannot be explained by hyperpolarization of the nerve terminals to complete block during the whole tetanization. Skoglund (1960 a) showed that the lack of PTP in the monosynaptic path of young kittens is due to hyperpolarization of the muscle afferents to complete block (see also Skoglund 1958), but this is apparently not the case with the efferents (see Nyström 1968 a and c). The afferent fibres are, however, known to be less mature than the efferent fibres for some time postnatally (Skoglund and Romero 1965).

Immature nerve fibres have long absolute and relative refractory periods (Hursh 1939, Skoglund 1960 b) and cannot, therefore, conduct impulses at high frequency. This probably explains why, in some kitten muscles, there is no major difference between decurarization obtained by high (250 c/sec) and low (45 c/sec) frequency tetanization (Fig. 3).

Since in the soleus and gastrocnemius of both kittens and adult cats decurarization is prominent, the respective end-plate potentials are likely to be increased post-tetanically. In the normal soleus of the adult cat this is probably the cause of the appearance of repetitive discharge. It is not, however, known whether an increase in the end-plate potentials is also necessary for the potentiation of that twitch potentiation which, in both kittens and adult cats, is not caused either by recruitment of muscle fibres or by repetitive discharge. In adult cat muscles a PTP has been obtained after direct stimulation (Brown and von Euler 1938, Bowman, Goldberg and Raper 1962, Standaert 1964) which does not include activation of the nerve fibres or their terminals. Consequently this PTP cannot be caused by any change in size of the end-plate potentials. Whether this type of PTP can be produced in kittens as well remains to be elucidated.

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References

BOWMAN, W. C., A. A. J. GOLDBERG and C. RAPER, A comparison between the effects of a tetanus and the effects of sympathomimetic amines on fast- and slow-contracting mammalian muscles. *Brit. J. Pharmacol.* 1962. *19.* 464—484.

BROWN, G. L. and U. S. VON EULER, The after effects of a tetanus on mammalian muscle. *J. Physiol.* (London) 1938. *93.* 39—60.

BULLER, A. J. and D. M. LEWIS, Further observations on the differentiation of skeletal muscles in the kitten hind limb. *J. Physiol.* (London) 1965. *176.* 355—370.

COLOMO, F. and P. ROCCHI, Staircase effect and post-tetanic potentiation in frog nerve-single muscle fibre preparations. *Arch. Fisiol.* 1965. *64.* 189—266.

FENG, T. P., L. Y. LEE, C. W. MENG and S.-C. WANG, Studies on the neuromuscular junction. IX. The after effects of tetanization on n-m transmission in cat. *Chinese J. Physiol.* 1938. *13.* 79—108.

FENG, T. P., T. H. LI and Y.-C. TING, Studies on the neuromuscular junction. XII. Repetitive discharge and inhibitory after-effect in post-tetanically facilitated responses of cat muscles to single nerve volleys. *Chinese J. Physiol.* 1939. *14.* 55—80.

GAGE, P. W. and J. I. HUBBARD, An investigation of the post-tetanic potentiation of end-plate potentials at a mammalian neuromuscular junction. *J. Physiol.* (London) 1966. *184.* 353—375.

HUGHES, J. R., Post-tetanic potentiation. *Physiol. Rev.* 1958. *38.* 91—113.

HURSH, J. B., The properties of growing nerve fibres. *Amer. J. Physiol.* 1939. *127.* 140—153.

HUTTER, O. F., Post-tetanic restoration of neuromuscular transmission blocked by d-Tubocurarine. *J. Physiol.* (London) 1952. *118.* 216—227.

LILEY, A. W. and K. A. K. NORTH, An electrical investigation of effects of repetitive stimulation on mammalian neuromuscular junction. *J. Neurophysiol.* 1953. *16.* 509—527.

LLOYD, D. P. C., Post-tetanic potentiation of response in monosynaptic reflex pathways of the spinal cord. *J. gen. Physiol.* 1949. *33.* 147—170.

NYSTRÖM, B., Mechanical and electrical responses to single shocks in developing cat leg muscles following tetanization. *Acta physiol. scand.* 1968 a. *74.* 207—225.

NYSTRÖM, B., Fibre diameter increase in nerves to "slow-red" and "fast-white" cat muscles during postnatal development. *Acta neurol. scand.* 1968 b. In press.

NYSTRÖM, B., Postnatal development of motor nerve terminals in "slow-red" and "fast-white" cat muscles. *Acta neurol. scand.* 1968 c. In press.

NYSTRÖM, B., Histochemical studies of end-plate bound esterases in "slow-red" and "fast-white" cat muscles during postnatal development. *Acta neurol. scand.* 1968 d. In press.

NYSTRÖM, B., To be published. 1968 e.

NYSTRÖM, B. and S. SKOGLUND, Neuro-muscular transmission in the kitten. *Acta Soc. Med. Upsalien.* 1965. *70.* 225—228.

SKOGLUND, S., The reactions to tetanic stimulation of the two-neuron arc in the kitten. *Acta physiol. scand.* 1960 a. *50.* 238—253.

SKOGLUND, S., The activity of muscle receptors in the kitten. *Acta physiol. scand.* 1960 b. *50.* 203—221.

SKOGLUND, S., Maturation of reflex activity. I. Neural interactions. The Interneuron. UCLA Forum in Medical Sciences. 1968. In press.

SKOGLUND, S. and C. ROMERO, Postnatal growth of spinal nerves and roots. *Acta physiol. scand.* 1965. *66.* Suppl. 260.

STANDAERT, F. G., The mechanisms of post-tetanic potentiation in cat soleus and gastrocnemius muscles. *J. gen. Physiol.* 1964. *47.* 987—1001.

WALKER, S. M., Action potentials in rat muscle with twitch tension potentiated by KCl treatment, adrenalectomy, tetanus and treppe. *Amer. J. Physiol.* 1948. *154.* 63—72.

WALL, P. D. and A. R. JOHNSON, Changes associated with post-tetanic potentiation of a monosynaptic reflex. *J. Neurophysiol.* 1958. *21.* 148—158.

Acta physiol. scand. 1968. 74. 238—245

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Effect of Potassium Magnesium Aspartate on the Capacity for Prolonged Exercise in Man

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Abstract

Ahlborg, B., L.-G. Ekelund and C.-G. Nilsson. *Effect of Potassium-Magnesium Aspartate on the capacity for prolonged exercise in man.* Acta physiol. scand. 1968. 74. 238—245.

The effect of potassium-magnesium-aspartate on the capacity for continuous prolonged standardized physical exercise (exercise times about 90 min) has been investigated in 6 normal young men. After administration of potassium-magnesium-aspartate the capacity for prolonged exercise increased about 50 per cent. Possible mechanisms behind this effect are discussed.

An effect of potassium and magnesium salts of aspartic acid on muscular fatigability has been demonstrated experimentally in animals by, among others, Laborit et al. (1958), Laborit (1961), Nakahara et al. (1964) and Tapin (1962). In man no similar objective registration of the effect on muscular fatigability has been described so far, only subjective estimates of performance (Tapin 1962).

Pronounced objective effects in animal experiments, good subjective effects in humans with different forms of fatigue (Kruse 1961, Taylor 1961, Shaw 1962) and lack of objectively demonstrable effect on muscle strength and other variables after potassium-magnesium-aspartate in humans (Consolazio et al. 1964) have been reported. It has therefore been considered to be of interest to investigate, whether a positive effect on the capacity for prolonged standardized physical exercise after oral administration of potassium-magnesium-aspartate can be objectively demonstrated.

Material

During 1963–1966 [illegible] 3[illegible] healthy [illegible] men have been examined [illegible] the Military Medical Examination Centre (MMEC), Karolinska sjukhuset, regarding their capacity for prolonged standardized physical exercise [illegible] constant load until physical exhaustion, each being tested more than 1[illegible] times (Ahlborg 196[illegible]).

Among these men 6 were [illegible] had [illegible] out [illegible] be especially fitted for this particular form of exercise. They were further examined three [illegible] Some data for the subjects are shown in Table I. As [illegible] Swedish standards they were all of about mean height and weight. 4 of [illegible] subjects were [illegible] and 2 [illegible] little below average physical fitness. They worked [illegible] of [illegible]

Methods

The subjects underwent certain preparatory examinations prior to the experiments. A *health history* was recorded. A *physical examination* was performed. *Height* and *weight* were measured. The *physical working* capacity was expressed as the amount of work that the subject could perform on bicycle ergometer at pulse rate of 170 beats/min, W170 (Sjöstrand 1960). All *pulse rates* were calculated from electrocardiograms, at least 20 heart cycles being counted for each value. The form of *prolonged exercise* chosen was continuous leg work on an electrodynamically braked bicycle ergometer (Holmgren and Mattsson 1954) with the subject in the sitting position and working at constant load. The loads were measured in kpm/min. The same bicycle ergometer with unchanged static dimensions was used for all tests. By way of pilot study suitable individual load was chosen so that physical exhaustion at the first exercise test should occur after about 90 minutes of continuous exercise. The exercise was not stopped by the subject until complete exhaustion and pain of increasing severity had been experienced in the leg muscles for few minutes. Current *statistical methods* were used (Snedecor 1959). Potassium-magnesium-aspartate[1] was given orally in an amount of 1.75 g every 6th hr (5 tablets×4).

Body weight was determined before and just after every exercise period. By total weight loss is meant the difference between weight before exercise plus weight of administered water during exercise and weight after exercise. During the prolonged exercise the pulse rat and respiratory rate were registered every second and tenth minute, respectively. During exercise water was given per os ad lib.

Procedure

On 4 consecutive days, which will be called day 1, 2, 3 and 4, prolonged exercise to exhaustion was performed every day beginning at 1 p.m. The subjects were not fasted. They did not eat standardized diet. On the other hand what they had been eating before and during the 4 days of the test was registered. It is known that very carbohydrate-rich diet in connection with physical training can increase the capacity for prolonged exercise markedly (Ahlborg et al. 1967). None of the subjects, however, had at any time during the test period eaten an especially carbohydrate-rich, or in any other way special, diet. Three days before day 1 the preparatory examinations were performed including W170 and pilot study concerning the suitable individual load for exercise during about 90 minutes until physical exhaustion. Beginning at 6 p.m. on the day before day 1, 5 tablets were administered every sixth hr. The last 5 tablets were given 1 hr before the prolonged exercise test on day 4 (see Fig. 1). All subjects were given placebo tablets before the tests on days 1 and 4. Before day 3 active substance was given. The subjects were told that the tests were aimed at elucidating the influence of vitamin tablet on maximal performance time. No information was given to the test supervisor (the same nurse on all days) or to the subjects as to when placebo or active substance was administered. The placebo and active tablets were identically looking. About 2 months later 2 of the subjects were tested in an identical manner but with the exception that now only placebo was administered.

Results

The results are presented in Table II and Fig. 1.

Between the mean work times (to exhaustion) on day 1, 2 and 4 there are certain numerical though not statistically significant differences. On day 3, before which active substance had been given during 19 hours, the mean work time is 128 min compared to 85 min on day 2. This increase of about 50 per cent is highly significant ($P<0.001$). The mean amount of work performed on day 2, 93,500 kpm, rose to 140,800 kpm on day 3.

The mean pulse rate after 10 min of exercise did not differ statistically on the different days. The mean pulse rat after 40 min of exercise on day 3, 151.8 beats/

[1] The preparation used was Trommcardin, every tablet containing 175 mg mono-potassium-D, L-aspartate and 175 mg mono-magnesium-D, L-aspartate) manufactured by Trommsdorff Chem. Fabrik, Aachen, Germany.

We wish to express our thanks for the kind supply of the drug.

TABLE II Some data t repeated prolonged exercise tests during 4 consecutiv days

Subject	Work load (kpm/min)	Duration (min)			
		Day 1	Day 2	Day 3	Day 4
1	1150	96	74	128	94
	1200	92	102	142	62
3	850	86	74	112	98
4	1150	10_	92	122	91
5	850	94	94	14_	92
6	1400	9?	76	122	90
x̄	1100	93 7	85.5	128.0	88.3
SD	214	5_	12.2	12.0	13.2
range	850—1400	86—102	74—102	112—14	62—98

Subject	Heart rate after 40 min exercise (beats/min)			
	Day 1	Day	Day 3	Day 4
1	154	156	147	150
2	169	167	168	165
3	149	141	146	144
4	150	156	146	151
5	154	160	140	146
6	168	174	164	168
x̄	157.3	159.0	151.8	154.0
SD	8.9	11.2	11.3	10 0
range	149—169	141—174	140—168	144—168

Subject	Heart rate the end of exercise (beats/min)			
	Day 1	Day 2	Day 3	Day 4
1	158	148	162	151
2	173	168	169	1_8
3	160	152	158	157
4	167	162	159	150
5	156	164	160	146
6	184	184	187	177
x̄	166.3	163.0	165 8	1_6.5
SD	10 7	12 7	11 1	11 0
range	156—184	148 184	158—187	146 177

sumption of energy of short duration the transport of oxygen (blood circulation) will limit the rephosphorylation of active phosphates. When the tore of gl ogen is emptied, the subject will be nabl to cont nue the work at the ctual work load This also means that the breakdown of gl cogen is one of the critical fa t rs for

Heart rate after 10 min exercise (beats/min)

Day 1	Day 2	Day 3	Day 4
153	149	148	152
158	158	157	160
135	139	140	138
148	140	141	146
148	148	140	144
158	155	152	150
150.0	148.2	146.3	148.3
8.6	7.7	7.2	7.5
135—158	139—158	140—157	138—160

Heart rate after 60 min exercise (beats/min)

Day 1	Day 2	Day 3	Day 4
156	159	160	145
174	165	165	156
162	150	155	147
158	153	147	148
156	161	154	146
170	180	174	175
162.7	161.3	159.2	152.8
7.6	10.6	9.4	11.3
156—174	150—180	147—174	145—175

Heart rate increase from 10 min till 60 min (beats/min)

Day 1	Day 2	Day 3	Day 4
3	10	12	—7
16	7	8	—4
27	11	15	9
10	13	6	2
8	13	14	2
12	25	22	25
12.7	13.2	12.8	4.3
8.2	6.2	5.7	11.4
3—27	7—25	6—22	—7—25

energy supply in the working muscle and that the speed of net breakdown of muscle glycogen will affect the working time. During submaximal prolonged exercise as in this study the lack of muscle glycogen most probably will be the limiting factor but the decrease of the respiratory quotient indicates that combustion of fat gradual-

Table II (forts.)

Subject	Respiratory rate after 10 min prol. exercise (breaths/min)			
	Day 1	Day	Day 3	Day 4
1	28	32	32	30
2	25	23	26	24
3	26	26	7	28
4	33	34	33	35
5	28	26	30	30
6	28	29	19	20
$\bar{x}$	28.0	28.3	28.2	27.8
SD	2.8	4.1	5.6	5..
range	25—33	23—34	19—33	20—35

ly contributes a greater portion of the energy supply. The same conclusion may be drawn from the slope of the muscle glycogen-time relationship shown by Hultman 1967.

Possible mechanisms behind the increased capacity for prolonged exercise after administration of potassium-magnesium-aspartate might be:

1. Accelerated resynthesis of glycogen in the muscles. It has been demonstrated that the speed with which glycogen is resynthesized can be accelerated by exercise plus a *carbohydrate rich diet* (*Ahlborg et al.* 1967, *Hultman* 1967).
2. A glycogen sparing effect. It is reasonable to assume that the capacity to perform a 50 per cent longer work on the same work load after administration of potassium-magnesium-aspartate is due to a decreased rate of glycogen utilization. This means that a smaller part of the energy during work is supplied from glycogen and a correspondingly greater part from fat.
3. Changed (accelerated) rate of resynthesis of phosphocreatine.
4. Potassium-magnesium-aspartate acts in an energy sparing way by improving the muscle efficiency so as to require less energy for more work. However unlikely this possibility has to be considered in view of the results reported by Nakamura-Haruo 1963 concerning the effect of potassium-magnesium-aspartate on oxygen consumption in rats and on the survival time of mice in hypoxia.

Investigations are in progress to evaluate these theories.

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References

AHLBORG, B. Capacity for prolonged exercise in man. *Försvarsmedicin* 1967. 3. Suppl. 1.

AHLBORG, B., J. BERGSTRÖM, J. BROHULT, L.-G. EKELUND, E. HULTMAN and G. MASCHIO, Human muscle glycogen content and capacity for prolonged exercise after different diets. *Försvarsmedicin* 1967. 3. 85—99.

Total weight loss during prol. exercise (kg)

Day 1	Day 2	Day 3	Day 4
1.4	1.3	1.7	1.7
1.1	1.6	2.2	1.1
1.0	0.9	1.4	1.3
1.3	1.8	1.9	1.4
1.4	0.9	2.2	1.1
1.0	1.7	2.7	2.4
1.20	1.37	2.02	1.50
0.19	0.40	0.45	0.49
1.0—1.4	0.9—1.8	1.4—2.7	1.1—2.4

AHLBORG, B., J. BERGSTRÖM, L.-G. EKELUND and E. HULTMAN, Muscle glycogen and muscle electrolytes during prolonged physical exercise. *Acta physiol. scand.* 1967 *70* 129—142.

CONSOLAZIO, C. F., R. A. NELSON, R. O. MATOUSH and G. J. ISAAC, Effects of aspartic acid salts (Mg and K) on physical performance of men. *J. appl. Physiol.* 1964 *19* 257—260.

EKELUND, L.-G. Circulatory and respiratory adaptation during prolonged exercise. *Acta physiol. scand.* 1967 *70* Suppl. 292.

HOLMGREN, A. and K. H. MATTSSON, A new ergometer with constant work load at varying pedalling rate. *Scand. J. clin. Lab. Invest.* 1954 *6* 137—140.

HULTMAN, E., Studies on Muscle Metabolism of Glycogen and Active Phosphate in Man with special reference to Exercise and Diet. *Scand. J. clin. Lab. Invest.* 1967 *19* Suppl. 94.

HULTMAN, E., J. BERGSTRÖM and N. McLENNAN ANDERSON, Breakdown and resynthesis of phosphorylcreatine and adenosine triphosphate in connection with muscular work in man. *Scand. J. clin. Lab. Invest.* 1967 *19* 56—66.

KRUSE, CH. A., Treatment of Fatigue with Aspartic Acid Salts. *Northw. Med. (Seattle)* 1961 *60* 597—603.

LABORIT, H. *Potassium Aspartate and Magnesium Aspartate fatigue recovery promoting process and compositions.* United States Patent Office 3 009859 — patented Nov. 21 1961.

LABORIT, H., R. MOYNIER, G. GUIOT, C. BARON and P. NIAUSSAT, Influence des différents agents pharmacologiques sur l'épreuve de nage du Rat blanc. *C. R. Soc. Biol. (Paris)* 1958. *152* 486—487.

NAKAMURA, M., S. YAMADA, H. SAKAMAKI, Y. NAKAMISHI, T. TOMIT and T. YOSHIHARA, Antifatigue effect of Potassium-Magnesium salt of aspartic acid on skeletal muscles and its mechanism of action. *Agr. biologie* 1964 *5* 245—255.

NAKAMURA-HARTO, Effect of Potassium and Magnesium Salts of L-Aspartic Acid on Blood Ammonia Levels, Oxygen Consumption and Tolerance Time in Hypoxia. *J. Physiol. J. p.* 1963. 25 150—154.

SHAW, D. L., Management of Fatigue—a Physiological Approach. *Amer. J. med. Sci.* 1962 *243* 758—769.

SJÖSTRAND, T. Functional capacity and exercise tolerance in patients with impaired cardiovascular function. *Clinical and cardiopulmonary physiology* N.Y. Grune & Stratton 1960. 201—219.

SNEDECOR, G. W. *Statistical methods.* The Iowa State College Press. Ames, Iowa, USA. 1959.

T... , M., *Activité Pharmacodynamique de l'Acide Aspartique ... le ... sodé.* Intérêt en clinique humaine dans le traitement de l'... du Myocarde. Thèse méd. (Bar-le-Duc) 1962.

TAYLOR, B. B. The Fatigued Worker. ... *Med.* 1961 2 533—538.

V..., C., *Contribution à l'étude de la fatigue ...neuro-musculaire et de son traitement chez les athlètes.* Thèse méd. (Paris) 1959.

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On the Physiological Significance of the Shape of the Endosomatic Galvanic Skin Reaction in the Cat

By

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Abstract

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Endosomatic galvanic skin reactions (GSR) have been evoked in cats by single or repetitive stimulation of the distal stump of sectioned sciatic nerve. The GSR were recorded with macroelectrodes from the surface of the central pad of the cat paw. The shape of the single GSR induced by single impulse is fairly constant in given preparation. The shape of the "fused" GSR induced by tetanic stimulation is, however, variable and generally intermediate between two extreme types, "slow" and "fast" type. A stimulation of low intensity and frequency and arrest of the regional blood flow causes the reaction to change in the direction of the "slow" type. The "fused" GSR induced after a period of rest often still deviates greatly from the mentioned extreme types. The shape of the ascending phase of the "fused" GSR is decisively determined by the frequency and intensity of the stimulation. The view is forwarded that the factors responsible for the observed systematic variations in the shape of the "fused" GSR may be those which cause changes in the amplitude of the single GSR with constant stimulus parameters and in the d.c. potential level of the skin. Changes in the impedance of the skin seem to play a central role in these mechanisms.

It has been found that the amplitude of the endosomatic galvanic skin reaction (GSR) is determined by, besides other factors, the intensity and frequency of the stimulation of the efferent postganglionic nerve fibers and that the intensity and frequency are at least qualitatively interchangeable (Lang 1968). Hence it may be assumed that the amplitude of the GSR is determined by the total number of unit potentials per time unit in the efferent nerve fibers that innervate the cells producing the reaction. One would hence expect that when the activity of these fibers is prolonged, the shape of the GSR varies in accordance with the changes in the time distribution of the efferent activity and correspondingly would remain constant when the number of unit potentials per time unit is constant.

The main purpose of the present study was to determine whether this hypothesis is valid. It was found that the shape of the GSR varied greatly when the period of stimulation was prolonged, although the stimulation parameters were held constant. It therefore became necessary to determine the reasons for the lability of the GSR

of longer duration. This study is a continuation of earlier investigations concerned with mechanisms determining the relationship between the endosomatic GSR and the d.c. potential of the skin and those determining the amplitude of the GSR (Lang 1967a, 1968).

Methods

The methods of preparing the cats and inducing and recording the GSR were the same as were used in the earlier studies (see Lang 1967a). Briefly, the stimulation was applied to the distal stumps of dissected sciatic nerves of anesthetized mongrel cats restrained with succinylcholine. Both the d.c. potential of the skin and the GSR were recorded from the central pad of the paw in question with d.c. coupled preamplifier and pen-recording polygraph. The employed electrodes were either metal (Ag-AgCl or Zn-$ZnSO_4$) macro electrodes or mercury calomel electrodes using potassium chloride solution as contact electrolyte. The reference electrodes were in contact with the tip of the tail. It was possible to record GSR simultaneously from two sites on the central pad and in this way to detect spatial differences in the reactions. The results are based on experiments conducted with 30 animals.

The time characteristics of the reactions were determined quantitatively by method described elsewhere (Lang 1967b). Briefly, the area below the recorded curve was measured planimetrically and the ratio of this area to the product of the peak amplitude and the speed of the recording paper was calculated. This ratio is quantity with the dimensions of time and is called the time index (TI) of the GSR. Its value is independent of the recording parameters (the amplification factor and speed of the recording paper) and represents the time of half-decay of the integrated and averaged GSR curv (right-angled triangle) which has the same area and the same height as the original response curve. The alue is given by A/hp sec., where A is the area below the (amplitude-time) curve in square centimeters, h the amplitude of the reaction in centimeters and p the speed of the recording paper in centimeters per second. Also the time indexes of the ascending and descending phases of the response curv can be separately determined in similar manner.

Results

A. *The S gle Reaction.* The GSR produced by a single supraliminal impulse is fairly constant in shape. Its most dominating component is a negative component with a fast ascending and an exponentially decreasing phase. The negative component is often followed by a slow component that varies in form but is usually positive in polarity. The amplitude of th slow component is usually only a small fraction of the amplitude of the negative and faster component when the prior basal activation has been sufficient (*cf.* Lang 1967a and Results B2).

The TI of the single GSR aries in different animals, but remains constant nd practically independent of the intensity of the stimulation in a given preparation. As a consequence, the TI of the reaction remains constant despite ev n large ariations in amplitude (Fig. 1).

B. *Th F sed Reaction.*

1. *General Characteristic.* Repeated stim la ion produced a fused GSR, the fusion being generally complet already when th timulation frequenc was imp/sec. The fused GSR aried in shape, however. A relativel short ascend ng phase preceded the peak in the begi ning of the curve which w followed by descend ng phase of arying form and h pe and after the timulation was disconti ued, by end phase which aried in duration and n which, as in the ase of the ingle GSR, there frequently was seen an overshoot below the initial pote tial level.

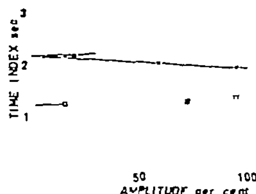


Fig. 1 *The time inde es (TI) f n gle GSR' of ary g amplitud* The different symbols mark mean values for reactions recorded from three different preparations using fou stimulation intensities the mean alues were computed from at least three individual observations. The greatest amplitude recorded from each preparation was taken to be 100.

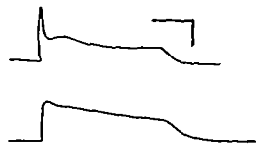


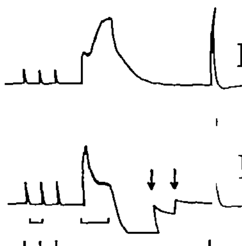
Fig. 2. *The f st (bac) d slow (below) type of fused GSR.* The reactions are from tw different preparations. The tetanisation (thick horizontal bar) frequencies were 24 and 18 imp/sec, respectively The stimulation intensity was supramaximal and the pulse duration 1 msec in both cases.
The calibrations are 5 mV and 10 sec

The potential curves were generally intermediate between the two extreme types shown in Fig 2. The first type the fast GSR" was characterized by a rapid rise to a maximum and then an initially steep fall and later a slow undulating decrease The second type "the low GSR was characterized by a slower rise to the maximum and a fairly slow linear decrease After a very long tetanisation period (sev ral minutes) however the potential gradually decreased to a constant level.

The amplitude of the single GSR produced by a constant stimulation intensity was generally *larger before than after a fused GSR. After* tetanisation, the amplitude of the single GSR increased slowly within 1—2 min, to the level before the tetanisation. The post-tetanic decrease n the amplitude of the GSR has been discussed in detail earlier (Lang 1968a)

2. *Th Sig f ca of Prior N al A t ity* In contra t to the decrease noted n the amplitude of the GSR fter tetanisation, there occurred a post tetanic increase in the amplitude whenever there had been a ufficiently long period of neural n-activity The negative d c potential component "the basal potential" recorded from the skin concurrently often ndergoes a hange usually a decrease in neg tivity (*cf* Lang 1967a These tw phenomena, the post tetanic amplitude ncre se *and the change in the basal potential when th repetitive timulation wa begun* explain why the GSR still deviated considerably from the extreme types *shown* in Fig 2. Two f sed GSR recorded from adjacent skin rea after a period of w k

Fig. 3. *Single and fused GSR' duced after period of rest* Both traces are simultaneous records from tw adjacent skin areas on the same central pad. The contact electrolyte (KCl) concentration was 1.0 N and 0.1 N for the upper and lower trace, respectively A period of inactivity of about 20 minutes preceded the stimulations. The stimulation intensity was supramaximal and the pulse duration 10 msec. The frequency of tetanization (thick horizontal bar) was 10 imp/sec. The d.c. potential level was adjusted to zero twice (arrow) during the recording of the lower trace the total shift was +12.8 mV
The calibrations are 2 mV and 10 sec. Note the change in the time scale when tetanization began.



activity before tetanization are shown in Fig. 3. In the upper trace the increase in the amplitude of the GSR is the only significant fact. As a consequence a pronounced rise occurs after the first peak in the early part of the fused curve. The curve recorded from the other skin area is largely determined by the change in th d.c. potential level, which was +12.8 mV The increase in amplitude was of the same order about 4.5 mV in both cases. The experimental conditions in these two cases did not differ except in the location of the recording lectrode and the concentration of the contact electrolyte. No systematic changes in the GSR were observed, however when these two factors were aried.

After a sufficiently high induced activ ty th amplitude of the GSR rises to a fairly constant value when the stimulation ntensity is held constant. Also the changes in the d.c. potential of the skin are relatively small after the activation. In order that the amplitude and shape of the reaction remain stable t is necessary that the *neuroeffector system has been sufficiently activated beforehand. The results de* scribed below were obtained when this condition was fulfilled.

3. *The Dependenc f th Shape of th GSR th St m l t on Par meter* The changes which the shape of the GSR undergoes w th increasing timulation intensity t constant stimulation frequency and with increasing stimulation frequency at constant stimulation intensity re shown in Figs. 4A and 4B (uppermost traces) respec tively In both cases the change is the same the shape of the curve changes from a slower type to the "faster type

The effects of a change in the stimulation parameters on the ascending phase of the GSR re more clearly seen in Fig. 5. The ascending phase becomes shorter both from left to right (stimulation frequency increases) and from the top to bottom

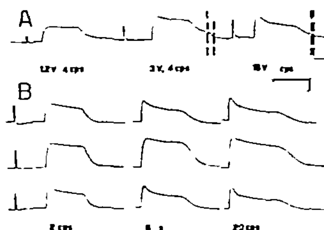


Fig. 4A. *The dependence of the shape of the fused GSR on the stimulation intensity.* The sciatic nerve was tetanized with the frequency and intensity given below each horizontal bar. The intensities of the single and repetitive impulses were equal and the pulse durations 10 msec. A section 10 sec long has been omitted between the broken vertical lines. The calibrations are 5 mV and 10 sec.

Fig. 4B. *The dependence of the shape of the fused GSR on the tetanization frequency. The influence of arresting the blood flow on the shape and amplitude of the fused GSR.* The sciatic nerve was tetanized with impulses of constant intensity and duration (as in the case of the single impulses) at the given frequencies. The three uppermost GSR were recorded before and the GSR in the middle during arrested arterial blood flow. The three lowermost GSR were recorded about 5 min after removing the clamp from the femoral artery. The calibrations are 5 mV and 10 sec.

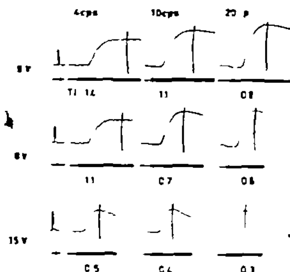


Fig. 5. *The effect of tetanization frequency and intensity on the amplitude and the shape of the ascending phase of the fused GSR.* The sciatic nerve was tetanized during the periods indicated by the thick horizontal bars with the given frequencies and intensities employing a pulse duration of 1 msec. The single GSR were evoked using the same pulse intensity and duration as in the horizontal stimulation series. The vertical lines are drawn through the maxima of the traces. The TI for the ascending phases of the GSR up to these lines are given below each trace.
The calibrations are 5 mV and 5 sec.

(stimulation intensity) increases. The relative shortening of the ascending phase with increasing stimulation frequency and intensity is of the same order in all stimulation series (see the values of the TI given below the curves).

Increase in the intensity or the frequency of the stimulation produces an equivalent change in the shape of both the total reaction and the ascending phase, i.e. the ratio of the area below the GSR curve to the peak amplitude decreases which means that the TI of the GSR diminishes.

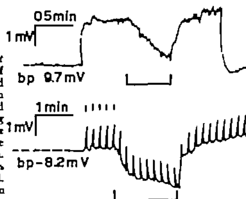


Fig 6. *Potential h ge sulting from arrest of the rterial circulation* The periods of stimulation of the sciatic nerv are indicated above the upper and the lower traces. I both cases the stimulation intensity slightly exceeded the threshold intensity I the upper recording the stimulation frequency slightly exceeded the fusion threshold (about 2 imp/sec) In the lower recording the frequency was 6 imp/min. The U-shaped line below each trace indicates the time when the femoral artery was clamped. The initial d c. potential level (bp) is given below each trace.

4 *Occlusion of the Regional Blood Flow and the Form f the GSR.* Occlusion of the arterial blood flow in the limb led to a typical change in the shape of the fused GSR. If the femoral artery was clamped a sufficiently long time before the stimulation, the reaction usually changed into one of the slow type, in which th ascending phase is longer and the slope of the falling phase is smaller than in the fused GSR obtained with unimpeded blood flow It was also often observed that when the stimulation parameters were held constant, the peak amplitude in eased after the artery was clamped. These changes were reversible the shape and ampli tude of the GSR reverted to the original after the blood flow was released (Fig 4B)

On using a stimulation of low frequency and low intensity a f sed GSR was obtained in which the amplitude no longer decreased after it had risen to ts peak alue. The potential decreased when the arterial circulation was arrested during the stimulation and rose again when the arterial clamp was removed (Fig. 6, upper trace) The change in the potential level corresponded to that which occurred in th skin basal potential following the occlusion of the blood flow but the amplitudes of simultaneously evoked single GSR did not, however change (Fig. 6, lower trace) Hence th change that occurred in the fused GSR when the artery was clamped for a short period was probabl due to a change in the d.c. potential level of the skin.

When the blood fl w was arrested for a longer period, say 15—20 min, the amplitude of the single GSR usually decreased at first. The decrease in the amplitude of the fused GSR c used by noxemia became evident later

Discussion

Patton (1948) has t ted that the neuroglandular delay and the time to peak of single GSR a re l nearly related in different preparations regardless of individual variations n th absol t alues When w ha e used the TI as measure of the time characteristics of the single GSR, w ha e found that it is constant and independent of the amplitude of the reaction I individual preparations. Hence when

the activation is effected by a single impulse, the neuroglandular delay the time to peak and the area-amplitude ratio of the GSR reflect an elementary autonomic process that is fairly constant in time possibly a change in the membrane permeability of the sweat duct wall cells (Shaver Brusilow and Cooke 1964)

The form of the GSR seems, however to be labile when tetanic stimulation is employed although the stimulation parameters are constant and hence the time distribution of the unit potential flow to the effector cells is constant. Apparently other less rapidly mobilized mechanisms become operative in the effector system in this case One may ask what these mechanisms could be

The fact that a plateau is not maintained by the fused reactions after the peak amplitude is reached has been ascribed to polarization of the skin associated with currents drawn in the GSRs (Patton, 1948) The slope and the shape of the descending phase can, however differ greatly in reactions having about the same peak amplitude. The shape of the reaction still depends decisively on the frequency of the stimulation though the maximal peak amplitude already has been reached. On the other hand, arrest of the regional blood flow is followed by both an increase in the amplitude and a slower potential decay (Fig. 4) These facts as well as the long post tetanic decrease in the amplitude of the single GSR indicate that a passive polarization of the skin cannot be solely be involved but that a physiological change occurs in the function of the effector system which persists for some time after the tetanic stimulation has ended.

When secretory potentials were measured with a micropipette electrode directly from the duct of a sweat gland, it was found that in response to repetitive stimulation the microelectrode held a uniform negativity for the first half minute and thereafter the potential began to decay toward the base line (Shaver Brusilow and Cooke 1962)

Although caution must be exercised when comparing results obtained with different techniques, it seems justified to assume that no decrease occurs in the output of the effector cells that produce the endosomatic GSR during a tetanisation period of 10 to 20 sec Hence the decay of the amplitude of the fused GSR that occurs during this period cannot be due to, for exampl exhaustion or adaptation of the effector cells or to a weakening of the neuroglandular transmission. It is f rther more unlikely that such exhaustion would be slower when the arterial circulation is blocked (*cf* Fig 4B

A theory based on the experimental findings ha been proposed according to which the negative component of the endosomatic GSR is produced in a circuit of resistive elements and unit potential generators, the latter situated in the distal parts of sweat ducts and coupled in parallel to each other and in series with th surface electrode (Lang 1968) According to this theory the amplitud of the GSR may diminish if the total resist nce mponent loading the un t potential generators and connected in parallel with th latter decreases. If th load were to ncrease even after the fused GSR ha re hed it peak mplitude the result would be a proportionate decrease in the potent l

It is possible to record, in addition to the endosomatic GSR, an exosomatic GSR, i.e. a decrease in skin impedance which has been attributed to the filling of the sweat ducts by sweat (Lloyd 1960) or an increase in the conductance of the epidermis (Edelberg 1961) If the potential and the impedance components were fully synchoronous, the increased loading of the unit potential generators caused by the impedance change would not distort the shape of the potential curve. However Lloyd has shown that the change in skin impedance is slower than the change in potential the time course of the former being greatly dependent on the stimulation parameters (Lloyd 1960) In addition, it has been found that there exists two asynchronous exosomatic GSR components in man (Edelberg 1966) Hence the reason for the decay of an endosomatic fused GSR following the initial rise to the peak may be a less rapid change in the skin impedance. The decrease in potential should be more abrupt and should take place earlier when the intensity and frequency of the tetanization are increased.

Another factor that may cause the endosomatic fused GSR recorded with a macroelectrode to differ in shape from the electrogram recorded with a micropipette electrode is a concurrent change in the d.c. potential of the skin. Skin areas containing sweat glands have been found to be negatively charged with respect to other skin areas or a subcutaneous reference point in the cat (Lang 1967a) The slow positive component of the endosomatic GSR may equally well be ascribed to a temporary decrease in this negativity: a considerable cumulation of the positive components has been observed on repetitive stimulation after a period of inactivity This decrease in the negative charge of the skin may also be a consequence of an increased loading of the current sources maintaining the negative d.c. potential. This also may arise from a decrease in the impedance of the epidermis (Lang 1967a) but also occlusion of the regional blood flow causes a slow change in the d.c. potential of the skin (*cf* Fig. 6) and it may be assumed that vasoconstriction induced by nerve tetanization causes a decrease in the negative d.c. potential level of the skin by the same mechanism as clamping of a large artery

The greatest variations in the shape of the endosomatic fused GSR become evident when stimulation occurs after a period of inactivity The changes may be primarily determined by large d.c. potential changes. On the other hand, a post tetanic increase is observed in the amplitude of the endosomatic GSR when the stimulation occurs after a period of inactivity This increment may be very large and has also been stated to be a consequence of a change in skin impedance (Lang 1968)

The fact that the ascending phase of the GSR becomes shorter when the intensity or frequency of stimulation increases may be explained by assuming that the effector system produces a maximum response more rapidly the higher the efferent nerve activity per time unit that reaches the effector cells It is also possible, however that the factors which cause the amplitude of the endosomatic GSR to decrease and the negative d.c. potential of the skin to diminish interrupt the ascending phase at an earlier stage when the stimulation becomes more intense

It has been found that the secretion of sweat diminishes before the amplitude of the GSR has undergone any greater decrease following the obstruction of the arterial blood flow of the limb (Lang and Lehtinen 1966). If, as Lloyd (1960) has stated, the impedance of the skin is largely determined by extent of filling of the sweat gland channels, we can in the light of the preceding observations understand the prolongation of the ascending phase, the increase in the peak amplitude and the slower decay of the endosomatic GSR after the artery is clamped.

We may summarize the above by saying that many factors are responsible for the differences between the form of the endosomatic GSR and that of the electrogram recorded with a micropipette electrode. The shape of the endosomatic GSR then depends on the relative importance of these factors. This implies also that no reliable conclusions can be drawn about the time characteristics of the neural activity that produces the GSR from the shape and the TI of the fused reaction curve recorded by the employed method.

References

Edelberg, R., Microelectrode study of the galvanic skin response. *Fed. Proc.* 1961, *20*, 245.

Edelberg, R., Response of cutaneous water barrier to ideational stimulation. *J. comp. physiol. Psychol.* 1966, *61*, 28—33.

Lang, A. H., Skin d.c. potentials and the endosomatic galvanic skin reaction in the cat. *Acta physiol. scand.* 1967, *69*, 230—241.

Lang, A. H., A mathematical definition of the tonicity of the galvanic skin reaction in the cat. *Acta physiol. scand.* 1967, *69*, 341—347.

Lang, A. H., On the physiological significance of the amplitude of the endosomatic galvanic skin reaction (GSR) in the cat. *Acta physiol. scand.* 1968, *73*, 151—160.

Lang, A. H. and I. Lehtinen, Sweating and the galvanic skin reaction: two independent functions of the sweat glands. *Acta physiol. scand.* 1966, *68*, Suppl. 277.

Lloyd, D. P., Electrical impedance changes of the cat foot pad in relation to sweat secretion and reabsorption. *J. gen. Physiol.* 1960, *43*, 713—722.

Patton, H. D., Secretory innervation of the cat's footpad. *J. Neurophysiol.* 1948, *11*, 211—227.

Shaver, B. A., S. W. Brusilow and R. E. Cooke, Origin of the galvanic skin response. *Proc. Soc. exp. Biol. (N.Y.)* 1962, *110*, 559—564.

Shaver, B. A., S. W. Brusilow and R. E. Cooke, Electrophysiology of the sweat gland: intraductal potential changes during secretion. *Bull. Johns Hopk. Hosp.* 1965, *116*, 100—109.

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From the Institute of Physiology University of Helsinki, Finland

Effect of the Left Atrial Pressure on Vagal Afferent Discharge from the Left Atrium, and on the Sympathetic Postganglionic Discharge to the Heart

By

M. O. K. Hakumäki

A linear correlation has been found between the pressure impulse of the v-wa e of the left atrium, and th number of B-type vagal afferent impulses from the left atrium, both within the fractions of one cycle, and in the different successive cycles. A positive inverse correlation exists between the ratio of the number of B and A impulses, and the number of sympathetic postganglionic impulses.

A linear relationship seems to exist between the left atrial volume and the average firing frequency of the B-type vagal afferents of the left atrium (Paintal 1953) Morover there is linear correlation between the increasing fractions of the pressure impulse of the v-wave and the number of vagal afferent B-type impulses of the left atrium in multifibre preparations during spontaneous and changed pressure conditions (Hakumäki *et al.* 1968) The pressure impulse (I) can be expressed by the equation $I = P_{-diff} \times dt$, where P_{-diff} = the pressure difference of -wave of the left atrial pressure curve. The question arose whether similar relationship obtains between different pressure curves under changing pressure conditions. I an approach to this problem, experiments were made on 12 open-chest cats, anaesthetized with hlorose-urethane mixture, and given artificial respiration. The intrathoracic multifibre preparations of the nerves were made on the left side, by the application of modification of the technique of Jarnch and Zotterman (1948) The intra-atrial pressure was measured by

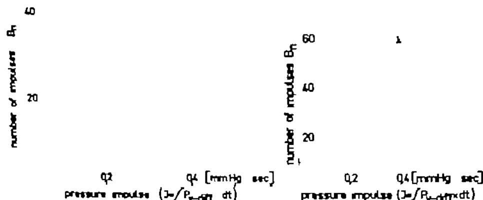


Fig 1 Fig 2

Fig. 1 The number of gal B-type impulses (B_n as f ion of he increasing pressure impulse (I) during tw different cy les, while blood was jec ed the lef atrium

Fig 2 The number of al B-type impulses (B_n) as fun o of the pressure impulse (I) in different successiv cycles while Ringer solution was injec d the femoral ein.

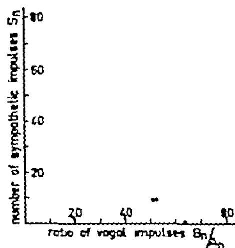


Fig. 3 The correlation between the ratio of the number of B and A impulses and the number of sympathetic postganglionic impulses (S_n) while blood was injected into the left atrium.

means of polyethylene tube inserted into the left trium through the pex of the auricular appendage and connected to a Sanborn pressure transducer m. 267 B. The hanges in the pressure and nerve impulses wer photographed at different phases of respiration, and during increase in the intra-atrial pressure by injections of Ringer solution into the femoral eln (10—15 ml in 8—10 sec or blood direct into the left trium (3—5 ml in 3—5 sec) The results are presented in Fig 1 and ... A linear correlation is pparent between the pressure impulse and the number of impulses, both within the fractions of one cy le and in the different successive cycles.

According to Millar and Biscoe (1965) a positive inverse correlation exists between the mean arterial pressure and the discharge of the splanchnic nerve Thus a tudy was made in ten cats similarly prepared whether the B-type impulses inhibit sympathetic efferentation to the heart. The sympathetic discharge was recorded from the inferior mpathetic cardiac nerve It was found that a rise in the number of the B-type impulses was followed by a partial, or even complete inhibition of the postganglionic sympathetic discharge to the heart. When the left atrial pressure was increased occasional atrial premature beats were observed, during which the atrial s tolic pressure was teeply elevated with an associated rise in the number of vagal A type impulses H wever no simultaneous significant reduction in the number of B-t pe impulses wa noted This phenomenon was always followed by a discharge of sympathetic impulses fter dela of 300—600 msec during the sympathetic silence

This seems to suggest that A type impulses activate the ympathetic system which is supported by the ratic f th number of B and A impulses being in positi e inverse correlation to the number of s mpathetic postganglionic impulses (Fig 3)

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References

HAKUMAKI, M O K R M BERGSTROM T H H or ... and H S S SAR J Vagal impulses from the trial stretch receptors in relation to in ra-atrial pressure S ad J h Lab I st 1968 64 Suppl 101
JARISCH A. and Y ZOTTERMAN Depressor reflexes from the heart. A t physiol scand 1949 16 31—3..
MILLAR, R. A. and T J BISCOE. Preganglionic sympathetic activity and the effect of anaesthetics. Brit J Anaesth 1965 37 804 -83
PARTRAL A. S. A study of cardiac and lef ral receptors J Physiol Lond. 1933 ?9 596-610

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Position and Velocity Sensitivity of Muscle Spindles in the Cat IV Interaction Between Two Fusimotor Fibres Converging on the Same Spindle Ending

By

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Abstract

LENNERSTRAND, G *Positio and velocity sensitivity of muscle spindles in th cat IV Inter ction between two fusimotor fibr s converging on the sam spindl end g* Acta physiol. scand. 1968 74 257—273

Position and velocity responses hav been measured during the co-activ tion of tw indentified fusimotor fibres influencing the same primary or secondary ending in the ankle extensor or flexor muscles of the cat. The effect of double-fibre activation has been compared with that of stimulating the participating single-fibres in isolation. During the activ tion of a dynamic static combination complete occlusion was usually encountered in the steady discharge at 'zero extension, in the position sensitivity and in the elocity responses to length decrement. Larger responses than any of the tw individual actions occurred in the response to dynamic length increment. In the dynamic-dynamic and in many of the static-static double-activations the responses were indistinguishable from those of single-fibre stimulation at higher stimulus frequency Other static-static combinations gav rise to 'averaging' of the two single-fibre effects this was seen f the position sensitivity and in the locity responses particularly during static I—static II co-activ tions Possible modes of fusimotor interaction in the spindle re discussed.

Several fusimotor fibres are known to influence the same ending (Hunt and Kuffler 1951 Crow and Matthews 1964 Bessou, Laport and Pagès 1966) They re probably concomitantly acti e in postural regulation as well as in the control of supraspinally organized movements. For example, in th intercostal muscles it has been shown that both dynamic and static fusimotor neurones contribute to the fusimotor control of primary endings in both reflex and respiratory movements (Corda, Euler and Lennerstrand 1966 Euler and Peretti 1966)

Knowledge of the combined action of fusimotor fibres converging to the same ending is therefore essential for the nderstanding of spindle f nction. A few reports on results of such experiments exist (Crow and M tthews 1964 Schafer and Henatsch 1968) but no sy tematic study has hitherto been performed. In the present stud combinations of dynamic and static fibres as well as of fibres of the same

kind have been examined. For practical reasons, only two fusimotor fibres were co-activated at a time both at the same rate of stimulation. The large number of combinations of fusimotor fibres and of stimulus rates available in these experiments would otherwise have rendered the analytical task overwhelming. The results show that in double fibre activation of an ending a full summation of the single-fibre effects seldom occurs neither with regard to the position nor to the velocity responses (see also Crowe and Matthews 1964). Based on characteristic effects of activating different combinations of fusimotor fibres some possible mechanisms of fusimotor interaction in a spindle ending are suggested.

Parts of the results have been preliminary reported elsewhere (Lennerstrand and Thoden 1967).

Methods

In an earlier paper (Lennerstrand and Thoden 1968a) a full description has been given of the general procedures and the technique. The methods for assessing position and velocity responses were also introduced in this paper.

For concomitant stimulation of two fusimotor fibres converging on the same ending, ventral root filaments containing one functionally isolated fusimotor fibre were placed on each of two pairs of platinum wire electrodes. The electrodes were connected to a stimulator either each pair at a time or both pairs at the same time. With this arrangement comparable responses of an ending to 'triangular' changes of length during fusimotor single- or double-fibre stimulation could be acquired within a short period of time. The accuracy of a comparison of double-fibre and single-fibre effects is therefore thought to be restricted mostly by the errors of measurements from the $f/\Delta L$ diagram, which were in the order of 5—10 %.

Results

For the determination of fusimotor effects the response in the non-activated state has been used as the reference level. The responses during single-fibre activation of the endings serve as controls for the evaluation of the action of double-fibre stimulation. Three kinds of net results could be distinguished, all of which are represented in Fig. 1 and 2. (i) the double-fibre response could be larger than any of the single-fibre responses. When, in such cases, both the single-fibre effects were of the same sign, the term summation has been used (e.g. Fig. 2, ending no. 5). When, on the other hand, the single fibre responses were of opposite signs, it seems adequate to think of the combined effect in terms of a potentiation of the positive effect by the other one (Fig. 2, no. 3). (ii) the double-fibre response could be equal to that of the strongest single fibre effect, in which case complete occlusion was at hand (e.g. Fig. 1, no. 1). (iii) the double fibre response could be smaller than that caused by the largest single fibre effect. In the latter instances, the combined effect attained a kind of average value in between those of the single-fibre activation (e.g. Fig. 1, no. 2b). If the two single-fibre values were of opposite signs, this double fibre effect could sometimes also be regarded as a partial summation (Fig. 2, no. 2).

It should be noted that these terms to some extent overlap conceptually. For example 'partial summation' and 'averaging' both imply a certain degree of occlusion in the double-fibre response.

Typical examples will be presented of the various effects of stimulating two fusimotor fibres to the same ending. However, the averaging of the results performed in earlier papers of these muscle spindle investigations was not pursued because the material collected on each combination was considered too small, particularly with respect to a complete dynamic analysis. The variety of effects within each type of combination and the existence of two control values rendered such an averaging unpractical. Another difficulty, which concerned the dynamic-static combination, was that the dynamically obtained response often seemed to be composed of parts deriving from different spindle components, so that no uniform mode of assessing the velocity response could be used (Lennerstrand and Thoden 1968*b*, *c*).

Position responses

Both the position responses to zero extension, or steady discharge rate, and the position sensitivity, *i.e.* the change in discharge rate with extension, have been determined.

Dynamic static. Such combinations occurred exclusively in primary endings, since dynamic fibres very seldom, if ever, influence secondary endings (Appelberg, Bessou and Laporte 1966). Eleven combinations of dynamic-static fibres were studied. In 5 the static fibres had type I effects on the position sensitivity and in 6 they had type II effects (for definitions of static type I and II effects see Lennerstrand and Thoden 1968*c*). In all cases except one, the position response to zero extension was of the type shown in Fig. 1, no. 1, which can be characterized as complete occlu

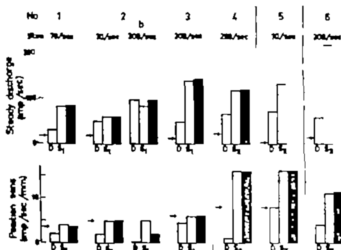


Fig. 1. Actions of combined dynamic-static activation, ... the rates of stimulation marked, on the steady discharge at zero extension (top) and on the position sensitivity (bottom) of six different primary endings. Values in the non-activated state shown by arrows. Result during single-fibre activation presented by open bars marked *D* for dynamic fibres and *S* for static fibres (I and II indicate the type of static effects). Values during combined activation represented by filled bars. Note ending no. 2 stimulated ... two diffe... rates.

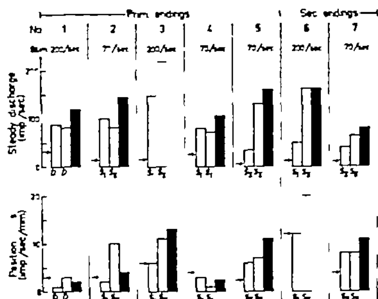


Fig. .. Action of dynamic-dynamic and static-static combinations on steady discharge and position sensitivity of primary (no. 1 to 5) and of secondary endings (no. 6 and 7). Results presented as in Fig. 1

sion. The effects of double fibre activation on the steady discharge rate of unit in F 1 re of particular interest. In this ending the largest position response on single fibre stimulation at 0 pulses sec was obtained with the static fibre (no. 2a) while at timulation with 200 hocks sec the d namic fibre was the most powerful (no. b). It is seen that at both rates of stimulation the effect on the position response of combined stimulation was to oc lude th weakest ingle fibre effect. The exceptional case w one in which the result was a partial summation (unit 6 in Fig. 1

Also the position sensitivity occlusion was the effect most commonly seen. This net result of d namic-static co-activation wa alw observed when static fibres with t pe II effects were inv l ed. In the d namic-static I ombinations tested three cases th positi n sensitivity tta ned an a erage lue between those of th ingle-fibre timulations. Examples of both t pes f doubl fibre responses are given in Fig. 1 Again different effects of different timulus rates is shown in ur t

In onclus on of the abov res lt the effect of d namic-static timulation on the pos tion response t zero extension and on th position sensitivit seems mostl t be one of occlusion

Dynamic-dynamic. F or th reason given I is this combination n be tudied only in primary endings. The t ad discharge of the tw endings which were b- jected to this kind f doubl f bre u ation sh wed a partial summation illustrated in Fig. 2, no. 1. In both ases the p tion sens tivit reached an a er e alue intermediate t those f single-fibre u ation

Static static. Both primary and sec ndary endings are influenced by tatic f

motor fibres (Appelberg, Bessou and Laporte 1966) Eleven static static combinations were studied in primary endings and nine in secondary endings. The subdivision of the static single-fibre effects into the types I and II has been maintained. In general the action on the position response of activation two static fibres were the same in both primary and secondary endings, and both kinds of endings will be treated together in the following. In all the combinations, except four a partial summation was seen in the steady discharge rate at static-static stimulation. Examples of these effects are given by units 2 and 3 in Fig 3 Unit 6 in Fig 2 is one of the exceptions in which double-fibre stimulation resulted in occlusion. Exceptional results were obtained three times in secondary endings and once in a primary ending. The static fibre combination in these exceptional cases were in the primary ending I—II and in the three secondaries one of each combination II—II I—II and I—I. Thus, no differences on the basis of type I and II effects of the participating static fibres could be revealed with respect to changes in position responses to zero extension during static-static co-activation.

On the other hand the position sensitivity seemed to be somewhat differently affected depending on the type of the static effects involved in the static-static combinations. When one fibre of type I and one of type II were co-activated, as done in eight cases, the position sensitivity mostly attained average values intermediate to those of the single fibre stimulations (Fig 2, no. ?) This action of double-fibre stimulation was always obtained when the static fibre of type I had the most powerful single fibre effect on the steady discharge rate. However when the type II dominated in a type I—type II combination, partial summation or sometimes 'potentiation (Fig 2, no. 3) in the position sensitivity was seen. Complete occlusion of the weakest single fibre effect has been observed once It occurred in both the steady discharge rate and in the position sensitivity The results acquired in four cases of type I—type I co-activation were roughly the same as those for type I—type II actions on the position sensitivity An example of 'averaging' of the single-fibre effects is shown in unit 4 of Fig 2. 'Occlusion occurred once also in this combination.

In eight type II—type II combinations, partial summation in the position sensitivity was by far the most common effect (Fig 2, no. 5 and 7) Averaging' was never seen. 'Occlusion was observed twice. In these cases occlusion occurred also in the steady discharge rate.

To sum up the results on static-static double fibre activation, some kind of summation of the in-going single-fibre effects was most commonly observed, either as a partial summation or an averaging' The latter net-effect was encountered only for the position sensitivity Complete occlusion was also seen when it occurred it was observed both in the steady discharge rate and in the position sensitivity

Velocity responses

The input signal for estimating velocity responses has been the step (or actually the square wave) changes in velocity generated in triangular changes of length (Len-

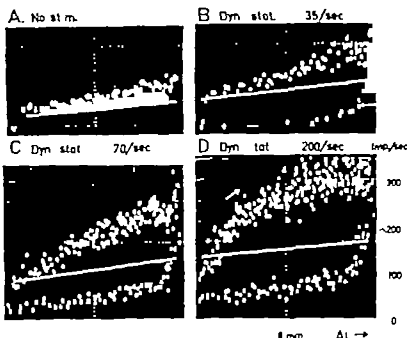


Fig 3 f ΔL diagrams of primary ending to 8 mm/sec 'triangular' movements. A Non-activated state B. During dynamic-static co-activation at 35 pulses/sec, C at 70 pulses/sec and D at 200 pulses/sec Dynamic and static single-fibre effects already presented (see text). Solid lines are approximate steady state curves Arrow shows the point movement of the plots in time Length (ΔL) increases to the right in the picture

...nerstrand and Thoden 1968a) The spindle ending responses to these muscle movements were recorded in f ΔL diagrams. The velocity response was subdivided into quick and a slow part as described previously by Lennerstrand (1968)

Dynamic static The effects of nine such fusimotor fibre combinations have been studied. In their general shape most of the f ΔL diagrams of primary endings subjected to dynamic-static co-activation were very characteristic. Usually the slope of the f ΔL curve in the phase of length increment corresponded exactly to that recorded during the dynamic activation alone In length decrement the slope coincided with that obtained at static activation. Furthermore the levelling sometimes seen in the f ΔL diagram during static fusimotor activation caused by 'driving' of the discharge (cf Lennerstrand and Thoden 1968c) persisted during combined activation in the part of the f ΔL curve recorded in length decrement In Fig 3 the typical f ΔL curves of dynamic-static co-activation at different rates of stimulation are shown. These particular dynamic and static single fibre actions have been fully presented in earlier publications (cf Fig 3 in the paper of Lennerstrand and Thoden, 1968b and Fig 10 in the paper of Lennerstrand and Thoden 1968c respectively) In this primary ending the position sensitivity was almost the same during fusimotor activation by the dynamic and the static fibres and also during co-activation of these two fibres Therefore the slow velocity response to length

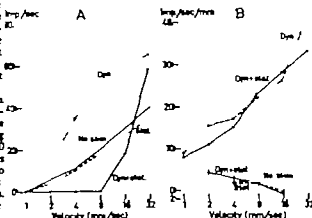


Fig. 4. Velocity responses of the primary ending in Fig. 3 in the absence and the presence of fusimotor single- and double-fibre stimulation. *A* 'Quick' component to length increment. *B* 'Slow' component to length increment (upper curves) and to length decrement (lower curves with crossed symbols). Both components of the velocity response plotted against velocity on log scale. Rate of stimulation 70 shocks/sec. Thin solid lines: values in non-activated state (only to length increment). Hatched lines during single-fibre stimulations. Thick solid lines during dynamic static co-activations.

increment was hardly changed in the combined activation in comparison to the value of dynamic single-fibre activation (Fig. 4*B*). In other endings the position sensitivity during the combined activation was increased above the value obtained during dynamic activation. Consequently the slow response was lowered, since the slope of the *f*/ΔL curve to length increment remained at the value of dynamic stimulation as just described. During length decrement the slow response was nearly always identical to that obtained during static activation (Fig. 4*B*) since [illegible] static fusimotor effects dominated both the position sensitivity and the dynamically obtained response to length decrement.

For a *f*/ΔL curve to length increment at combined dynamic-static activ[illegible] become equal in slope to that of the dynamic fibre activation alone it seemed [illegible] sary that the dynamic fibre at single-fibre stimulation elicited a higher [illegible] pulse frequency than the static fibre. If this was not the case, as in Fig. [illegible] fibre contribution dominated the whole *f*/ΔL curve during the combined [illegible] (see Fig. 5*D*). Out of nine dynamic-static combinations, dominan[illegible] fusimotor component in the response to length increment was seen [illegible] all of which employed static fibres with type II effects. The quick and [illegible] were in these instances equal to those acquired in the static single-[illegible] the ending.

However, with regard to mean impolse frequency during the phase of length [illegible]crement, the correspondence between different parts of the *f*/ΔL curves at double fibre and single fibre activation was less evid[illegible]t. When the dynam[illegible] effect dominated the response to stretch, the mean frequenc[illegible] during combined [illegible] usuall[illegible] increased to a value above that of pure dynamic activation (Fig. 5*B*, *D*; to be compared with Fig. 3*B*—*D* of Lennerstrand and Thoden 1968*b*). However the shift never became so large as to compensate fully for the increase [illegible] response by the combined stimulation. The quick response to length increment therefore was generally lower at combined stimulation than at dynamic [illegible]

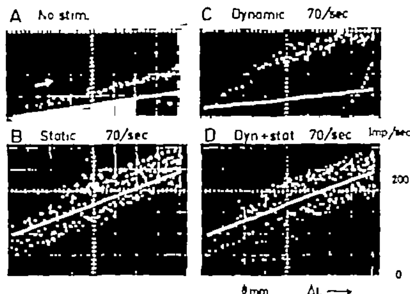


Fig. 5. *f* ΔL diagrams of primary ending to 8 mm/sec changes of length. A Passive ending. B. During static single-fibre activation. C. During dynamic single-fibre activation. D. During concomitant stimulation of the dynamic and the static fibre. Rate of stimulation in all cases 70 pulses/sec. Markings as in Fig. 3.

stimulation. Sometimes it even attained values below those of static activation. This is illustrated in Fig. 4 I. When the static fibre effect dominated the response to length increment during combined activation, no change in mean frequency was observed between the static single fibre and the dynamic-static *f* ΔL curves (Fig. 5*B* and *D*). In length decrement the mean frequencies of the *f* ΔL curves usually remained at the values obtained during static activation alone (Fig. 5*B* and *D*). In this respect the responses of Fig. 3 showing depression of the mean frequency are exceptions to the rule.

To sum up the action of dynamic-static activation on the quick and slow velocity responses to length increments were in most cases an averaging of the single-fibre effect in the velocity responses to length decrement an occlusion of the dynamic fusimotor single-fibre effect was most commonly encountered. It seems, however, as if the averaging effect on the different components of the velocity responses to length increment had no influence on the shape of *f* ΔL curve obtained in this part of the triangular movement. With regard to the slope the curve recorded during double fibre activation corresponded exactly to that during dynamic single fibre activation, provided this fibre produced the highest mean frequency to length increment at single fibre stimulation. Dominance of the static single fibre effect always resulted in an occlusion of all dynamic fusimotor influence as judged from the *f* ΔL curves during combined activation.

Dynamic-dynamic. The effect of concomitant stimulation of two dynamic fibres to a primary ending was similar to that of increasing the rate of dynamic single-

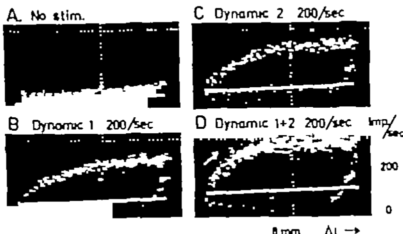


Fig. 6. f-ΔL diagrams of primary ending t 8 mm/sec length changes. Dynamic-dynamic combination. Ending without fusimotor activation in A actn ted by first dynamic fibre in B by second dynamic fibre in C nd by both fibres in D Activation t 200 shocks/sec. Markings as in Fig. 3.

fibre stimulation. The single-fibre effects were partially summated. This is seen in Fig 6, which presents f ΔL diagrams from a dynamic-dynamic experiment and in Fig. 7 where the values are plotted for the quick and the slow elocity responses and for the 'frequency ceiling' obtained in the same experiment.

Static static Ten combinations of this kind have been tested in primary endings and eight in secondary endings. Partial summation or 'a eraging of the single fibre effects was usually the result. Complete occlusion was ery rarely seen. Representa tive responses m f ΔL diagrams are shown in Fig 8 for a primary ending when stimulating a type I—type II combination, and in Fig 9 for a secondary ending with a type II—type II combination As a rule the effects on the elocity responses by

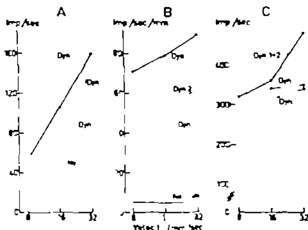


Fig. 7 'Quick elocity responses (A) 'slow velocity responses (B) and 'frequency ceiling (C) to length increases of the primary ending in Fig. 5 Values during combined activation to be compared with those of single-fibre stimulation and of the passive ending (only in A and B Rate of stimulation 200 shocks sec in all activations. Lines and labelling as in Fig. 4 A

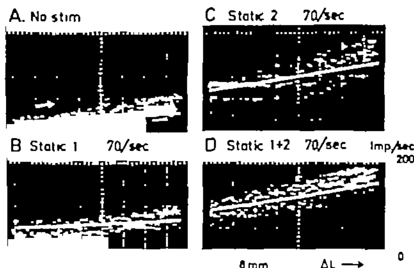
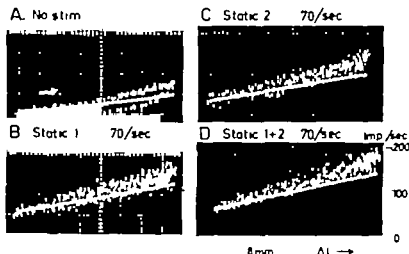


Fig. 8. f-ΔL diagrams f primary nding to 8 mm/sec ariations in muscle length. Fresh ending in A Static single fibre type I activation i B single-fibre type II activation in C and combined actis tion D Activation t 70 pulses/sec in ll cases. Not driving in C Markings as in Fig 3



F g 9 f ΔL diagrams of secondary ending t length hanges of 8 mm/sec Static II—II activation t 0 pulses sec A No stimula ion B Stim la ion of first static fibre alone C of second fibre lone and D of both fibres oncomi tantl Markings as in F g 3

each static double-fibre activ tion wa the same with regard to the type (i.e partial summation, averaging or ompl t occlusion) as the one obtained in the position sens th ity witl the same ombination This i diagr mmatically shown in Fig 10 where the quick and the l ow locit responses d ring single- and double-fibre activation are plotted f r the ending of Fig) In this ending partial summation of the teady discharge t nd the position sensiti ity resulted from the

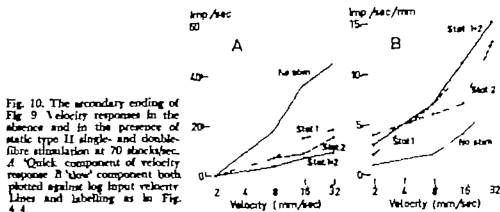


Fig. 10. The secondary ending of Fig. 9. Velocity responses in the absence and in the presence of static type II single- and double-fibre stimulation at 70 shocks/sec. *A* 'Quick' component of velocity response. *B* 'slow' component both plotted against log input velocity. Lines and labelling as in Fig. 4 4.

static-static co-activation. The same effect was observed on the quick (Fig. 10*A*) and the 'slow' velocity responses of the ending (Fig. 10*B*). In the primary ending of Fig. 8, where the position sensitivity was averaged, double-fibre activation had this effect also on the 'slow' velocity response. A quantitative presentation of the quick response was not made since these velocity responses were measured differently in passive primary endings and in endings activated by static fibre stimulation. However, expressed in absolute values, the quick velocity response at combined activation (Fig. 8*D*) is intermediate to those obtained in single-fibre activation (Fig. 8*B* and *C*).

It can also be noted that the 'driving' of the discharge in Fig. 8*C* on stimulating in isolation the static fibre of type II disappeared at the double fibre activation in Fig. 8*D* although the pertinent impulse frequency was represented also in the latter recording. It may also be mentioned that at two instances of activating a secondary ending by a type II—type II combination at the rate of 200 pulses/sec, the ending was unable to deliver a regular high rate of discharge in long sequences of the records every second action potential dropped out at impulse frequencies above 250/sec. This was never observed in primary endings, in which discharge rates could be maintained at the high values of about 400 imp/sec for quite long periods of time.

Analysis of dynamically obtained responses during spindle activation by succinylcholine

If the method outlined above for distinguishing dynamic from static fusimotor influence on primary endings is as valid during more complex patterns of spindle excitation as it is on activating only two efferent fibres, it might be a useful tool in analysing static and dynamic fusimotor contributions to reflex and other movements. In order to test this possibility, spindles were excited by succinylcholine, a competitive acetylcholine inhibitor with a strong activating effect on the spindle discharge (Granit, Skoglund and Thesleff 1953. For further references see Rack and Westbury

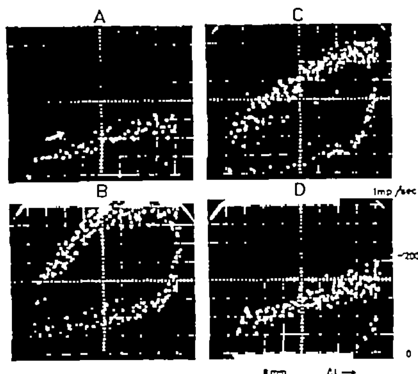


Fig 11 Excitation of a primary ending by succinylcholine f ΔL diagrams t 8 mm/sec length anges before A and t min B 4 min C) and 6 mi (D) after the injection of the e Markings explained in F 3

1966 Primary endings ha e been found to be affected for a longer period of time than secondary endings (Fehr 1963 Rack and Westbury 1966) and the most long-lasting excitation of the primary endings is reported to have the characteristics of d namic fusimotor activation on the response to stretch (Rack and Westbury 1966)

With th present technique further details could be revealed in the responses of primary endings to length changes d ring activation by succinylcholine In Fig 11 are presented the f fL diagrams t triangular length changes of 8 mm sec before (A) and at min B 4 min C and 6 min D after an intra enous injection of 300 μg kg b w t uccin lcholine Based on the slopes of f IL curve obtained in length changes f three different elocities separation could be made of two parts equivalent to those f und in primary endings activated by a dynamic-static combi nation (cf Fig 3 The activation of the tatic fus mot r component by the drug was seen to last sl rter than the acti ation of the d namic part 6 min after the injection no static effect could be detected nl the dynamic fusimotor action per sisted (Fig 11D) Th res lt extend those f Rack nd Westbury (1966) in that a hort-lasting static fus mot r influence wa clearl demonstrated in add tion to th dynamic effect

Discussion

The only reports available on dynamic responses to simultaneous activation of two functionally identified fusimotor fibres converging to the same ending are those of Crowe and Matthews (1964) and Schäfer and Henatsch (1968) They describe a few experiments performed in primary endings activated by one static and one dynamic fibre. Information on these matters is badly needed for the understanding of the fusimotor control in different reflexes as well as in centrally induced movements (*cf* Jansen 1966) The usefulness of the present method is illustrated by the experiments on spindles activated by succinylcholine The static and dynamic fusimotor contributions to the activation of the primary endings could easily be distinguished. Such qualitative tests seem most important at the present when still so little is understood about the function of fusimotor control.

Even if the present results are not nearly extensive enough for a quantitative description of the effects of combined fusimotor stimulation on the position and velocity sensitivities of spindle endings, they are though to be adequate to determine the principal modes of interaction between several fusimotor fibres influencing the same ending On these grounds they may be of value for a later analogue simulation of the spindle. Some common features existed within the different types of combinations on which the further discussion on the interaction could be based. During activation of two fibres of the same functional type, i.e. dynamic-dynamic or static-static combinations of fibres, the effects on position and velocity responses mostly came out as a partial summation or as an "averaging" of the responses during single-fibre activation. Both these net results of double fibre activation may reflect the same type of interaction one fusimotor fibre modified the effect of the other because they share at least one site of action in the spindle This interaction may also be regarded as occlusion of one fusimotor action by the other According to Crowe and Matthews (1964) and Schäfer and Henatsch (1968) combined stimulation of a dynamic and a static fibre resulted in partial summation of the individual effects both with regard to the static response and the mean impulse frequency in the response to dynamic stretch. This is not entirely in agreement with the results of the present work in which partial summation of the position response to zero muscle length at combined stimulation was only exceptionally observed. It was also found in the present study that the dynamically obtained f–ΔL responses to length increment or decrement at combined stimulation corresponded in shape exactly to the f–ΔL response of the single-fibre stimulation yielding the highest mean frequency during that phase of the movement When the static fibre was the dominating one in the combination, it completely occluded the dynamic fibre and the mean frequency of the response to combined stimulation was mostly exactly the same as in static activation alone However partial summation usually occurred in the response to length increment at the dynamic-static stimulation, if the dynamic fibre gave the biggest of the single-fibre responses during length increment.

When trying to explain these findings two possible modes of interaction be

tween individual fusimotor effects have to be considered. Firstly the two fusimotor fibres may innervate the same intrafusal muscl fibre on a mechanical basis combined stimulation might be expected to result in a full or partial summation of the individual responses. Secondly the fibres may innervate separate spindle structures in acting on different receptor terminals of an ending the responses deriving from separate receptor sites would then be electrically integrated into the final impulse frequency of the common afferent fibre The mechanical and the electrical type of interaction may well be at play simultaneously in the same ending. In anatomical and physiological studies fusimotor fibres have been found not only to innervate a single intrafusal fibre by multipl neuromuscular junctions (Kuffler Hunt and Quilliam 1951) but also to send nerve branches to several intrafusal fibres in the same (Adal and Barker 1965) and other spindles (Hunt and Kuffler 1951)

An explanation of the complete occlusion merely on the mechanical type of interaction would have to be based on the possibility of unloading one of the spindle components by contraction of other intrafusal fibres. On stimulating nerve fibres that influenced solely the nuclear chain fibres in an isolated muscle spindle Boyd (1966) observed a kinking of the nuclear bag fibres. In the dynamic-static coactivations, when complete occlusion was most often seen in the steady discharge at zero extension and in the position sensitivity the dynamic fibre activation still contributed to the dynamically obtained response The effect of unloading would be to move the nuclear bag fibres, which are the ones most likely to mediate dynamic fusimotor effects, into a lower range of extension. This would most probably not ffect the shape of the / ΔL curve of the nuclear bag component since position and velocity sensitivities are known to be uninfluenced by variations in the extension range (Lennerstrand and Thoden 1968b) but merely shift the curve to lower impulse frequency values. The responses to movements during combined activation would then ha e resulted from a summation of the responses of the nuclear chain and the unloaded nuclear bag component. The curve would take lower values than that in a summation of the responses of single-fibre stimulation. This was also seen in most of th curves to length increment.

However other part of the d namically obtained results on dynamic-static ac t vation could hardl be explained only by the hypothesis of unloading Firstly the slope of the / ΔL diagram to length increment was always found to be identical to that of the most powerful single-fibre response This would probably not occur so regularly if nuclear bag and chain responses lways summated. Secondly under these conditions the curve recorded during length decrement would not have taken exactly the same course as that during tatic single-fibre activation but should be shifted to lower values Therefore a closer examination of the possible modes of electrical integration of th resp nse arising in different receptor ites of the same ending seems necessary in order to explore the possible explanations of complete occlusion

Integration of the electrical events may be carried out in different ways depending on whether at the point of confluence the signal is still in the form of receptor

currents or whether at that point the modulation into impulse frequency has already taken place. In the case of integration of pulse coded signals it seems unlikely that the refractoriness of the afferent fibre alone determines the final discharge rate. This is because the impulses in the afferent discharge of both primary and secondary endings are rather regularly spaced in time at steady state and during dynamic length changes. Other mechanisms may be utilized when pulse-coded signal meet in afferent fibres of sense organs. By antidromic invasion and subsequent depression of the excitability of its fellow receptor sites the terminal in the receptor field eliciting the highest discharge rate to the common stimulus may entirely dominate the final afferent discharge. This mechanism, which can be regarded as a kind of lateral inhibition, is at work in tactile sensory units in the skin of the toad (Lindblom 1958) the cat and the monkey (Lindblom and Tapper 1966) In fact, the afferent discharge in the tactile units takes exactly the rate of the receptor initiating the highest impulse frequency

If this type of integration of pulse coded signals should be used to explain the 'occlusion' of one of the fusimotor effects, as it is seen e.g. in some of the dynamic-static fusimotor co-activations, it must be certified that impulses can be fired in the nerve branches from the receptor terminals to join the common afferent fibre of the ending. This hypothesis has not been physiologically proved but does not seem unlikely from an examination of available anatomical pictures (e.g. the papers by Barker 1948 and Boyd 1962) which show that the terminal afferent branches are myelinated and in many cases have at least one node interposed between the terminal site and the point of confluence in the afferent fibre.

Thus, in summing up the discussion on fusimotor interaction so far the partial summation and the 'averaging' of individual responses most often observed during the combined stimulation may originate either from mechanical interaction between the fusimotor effects of two efferent fibres impinging on the same intrafusal fibre, or from electrical interaction between receptor currents arising in intrafusal elements separately affected by the fusimotor stimulation. Complete occlusion on the other hand, which was often experienced in dynamic-static co-activation, may depend on an integration of pulse coded signals from the receptor sites, by which the terminal with the highest impulse frequency determines the rate of firing in the common afferent fibre of the ending

Probably all the modes of interaction suggested can be expected to be at work at the same time in setting up a response of an ending during double fibre activation. An indication that this is so would be the increase in mean impulse frequency to dynamic length increment above the value during dynamic single-fibre activation seen in dynamic-static activation. This might depend on contribution of static fibre activation in the response to length increment by any of the possibilities available for partial summation. The same would be the case for the dynamic fibre activation when the mean impulse frequency during length decrement at some dynamic-static double-fibre activations was decreased below the value obtained at static single-fibre activation.

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From the Department of Physiology University of Göteborg Sweden

The Origin of a Descending Pathway with Monosynaptic Action on Flexor Motoneurones

By

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Abstract

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The postsynaptic effect evoked lumbar motoneurones were studied following electrical stimulation of the brain stem in the cat The spinal cord was transected at the lower thoracic level leaving only the ipsilateral ventral quadrant intact. With stereotactical method low threshold focus was found in the medial brain stem from which monosynaptic EPSPs could be evoked in flexor motoneurones It is concluded that this effect is mediated by fibres descending in the ipsilateral medial longitudinal fascicle and it is tentatively suggested that these fibres originate from the ipsilateral upper medullary or lower pontine reticular formation. Monosynaptic EPSPs were also evoked some extensor motoneurones from this medial brain stem region and at such strength of stimulation that stimulus escape to the lateral vestibulospinal tract is excluded

Both extensor and flexor lumbar α-motoneurones receive monosynaptic EPSPs of supraspinal origin Lund and Pompeiano 1968) In this former investigation it was also shown that vestibulospinal pathways originating from the ipsilateral Deiters nucleus evoke monosynaptic EPSP exclusively in some species of extensor motoneurones and that flexor motoneurones receive inhibitory actions from the same pathway It was also concluded that the pathway with monosynaptic excitatory action on flexor motoneurones originates from a supraspinal structure outside the ipsilateral vestibular complex. The aim of the present experiments was to establish this origin. A preliminary report has been given (Grillner and Lund 1966)

Methods

The experiments were performed on 13 cats Ether anaesthesia was used during the surgical procedures. During the recording anaesthesia was maintained by injection of chloralose (70 mg/kg) or Nembutal 30 mg/kg The hindlimbs were denervated and a number of nerves on the left side mounted for stimulation Lower lumbar laminectomy was performed leaving dorsal and ventral roots intact, the latter in order to allow identification of motoneurones by their antidromic invasion. Intracellular recording was made with microelectrodes filled with 3 M KCl or 2 M K-citrate After low thoracic laminectomy the ventral and lateral funiclets, contralateral to the motoneurones investigated were transected at Th 10 and the dorsal

columns were removed between segments Th 10 to Th 12. Stimulating electrodes were placed on the ipsilateral thoracic spinal cord rostral to the contralateral lesion and in tw experiments a further lesion was made in the ipsilateral dorsal quadrant caudal to the stimulating lectrodes. After ernal cerebellectomy exposing the bottom of the fourth entricle, thin tungsten electrodes, insulated but for the tip (resistance 25—100 KΩ) were inserted in the brain stem under ocular control by means of a Horsley-Clarke instrument. Square wav pulses, 0.2 msec duration, were pplied to the brain stem (between tungsten lectrode cathode and an indifferent lectrode in the neck muscle) All other stimuli used were condenser discharges with half decay of 45 µsec. Descending olley and olleys in the dorsal roots were recorded from the cord dorsum by ball-tipped electrode t the L7 dorsal root entry zone against an indifferent electrode in the back muscles. The animals were curarized with gallamine triethiodide (Flaxedil® May and Baker Ltd.) and artificially respired with mixture of 6 % CO_2 in O_2. The arterial blood pressure was recorded continuously during the experiments. Blood loss was substituted by intravenous administration of mixture of high and low molecular dextran. Paraffin pools covered the spinal cord and the nerves mounted for stimulation. The temperature in these pools and the rectum was kept within 37—38 C. At the end of the experiments an lectrolytic lesion was performed by passing current through the brain stem electrode The brain stem was fixed in 10 % formalin, embedded in paraffin, and serial sections 20µ thick were made and stained with the Nissl technique to relate the position of needle tracks to the electrolytic lesion. In projection apparatus pictures were drawn from these sections to obtain maps as shown in F g 3 and 5. Spinal cord lesions were examined by similar methods or in wet and unstained serial sections cut with a freezing microtome.

Abbreviations PSP postsynaptic potential EPSP excitatory postsynaptic potential IPSP inhibitory postsynaptic potential PBSt, posterior biceps-semitendinosus ABSm, anterior biceps-semimembranosus G-S, gastrocnemius-soleus i, ipsilateral co, contralateral, VLF entral and lateral funicles; VQ, entral quadrant BS brain stem MLF medial longitudinal fascicle L, lumbar Th, thoracic; T threshold.

Results

1 *Flexor motoneu ones*

Only those motoneurones in which a monosynaptic EPSP could be evoked on stimulation of the ipsilateral ventral and lateral f nicles (VLF) at th lower thoracic level (*cf* Lund and Pompeiano 1968) were selected for further testing of effects evoked by electrical stimulation of the brain stem. The records in Fig. 1 are from a PBSt motoneurone. A shows a homonymous group Ia EPSP and B the antidromic invasion at a stimulus trength supramaximal for the motor axon. C shows the response evoked from the lower thoracic region with th early part of the EPSP being monosynaptically transmitted (Willis *et al* 1967 Lund and Pompeiano 1968) D shows the effect of single shock stimulation in a dorsomedial part of the brain stem ipsilateral to the motoneurone. 6.5 mm rostral to obex. The segmental latency (*i.e* the latency between the peak of the first positive potential in the L7 dorsal root entry zone lead and the onset of the EPSP) is 0.6 msec A comparison with the central latency for the group Ia EPSP in A gives the same alue Thus t is concluded that the EPSP in D is monosynaptically evoked The amplitudes of the monosynaptically evoked EPSP in C and D re about the same This confirms the concl sion (Lund and Pompeiano 1968) that any major contribution of propriospinal fibres above the lower thoracic level can be excluded All 29 PBSt motoneurones tested showed these characteristics with a segmental latency on th average 0.6 msec varying between 0.4—0.9 msec the longer latencies being mainly from more rostral regions.

Calculations of the conduction velocit for the f bres responsible for the mono-

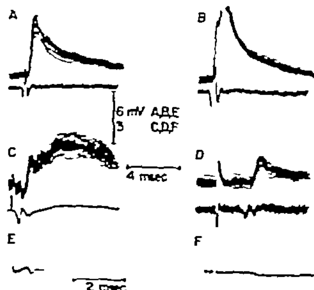


Fig. 1. In this and following figures upper traces in each record are recorded by the microelectrode negativity of which is signalled downwards. Records from a PBSt motoneurone. A and B show a group Ia EPSP and the antidromic invasion respectively on stimulation of the nerve to the PBSt muscles. C shows the effect evoked on supramaximal stimulation of the IVQ at lower thoracic level with the extracellular field potential, obtained at the same stimulus strength, in E. D shows the EPSP evoked from the ipsilateral brain stem 6.5 mm rostral to obex 0.5 mm lateral to the midline and 2 mm below the bottom of the fourth ventricle at a stimulus strength of 0.10 mA, with the extracellular field potential in F. Lower traces (A—D) are recorded from the L. dorsal root entry zone in this and the following figures (positivity of the central electrode being signalled downwards). Time calibration at E refers only to this record. Voltage calibration refers in this and the following figures to microelectrode recordings.

synaptic EPSP taking the peak of the first positive potential in the cord dorsum lead as a sign of the arrival to segmental level of the fastest fibres, gives about 120 m/sec independently of whether the lower thoracic spinal cord or the brain stem is stimulated. This value corresponds well with that earlier reported (Lloyd 1941, Magni and Willis 1963, Lund and Pompeiano 1968).

The threshold for the brain stem stimulation evoking monosynaptic EPSPs in flexor motoneurones was frequently below 0.01 mA. Fig. 2 shows the effect of increasing stimulus strength on the response in a PBSt motoneurone. It appears that the EPSP is saturated at 2.5 times threshold (Fig. 2 D). Careful examination of the record also reveals that the amplitude of the EPSP grows in parallel with the growth of the first positive potential of the descending volley recorded at the dorsal root entry zone. This suggests that the fibres giving rise to these two potentials are within the same fibre group.

The following experiments were performed in order to outline the extent of the brain stem region from which monosynaptic EPSPs could be evoked in flexor motoneurones. The electrode was inserted in parallel tracks 1 mm apart in different regions of the brain stem. In each track the threshold was determined at every mm to a depth of 5—6 mm below the bottom of the fourth ventricle. Fig. 3 shows the result of an experiment with tracking in a transverse plane 6 mm rostral to obex and recording from a PBSt motoneurone. The figures indicate multiples of the lowest threshold (0.01 mA). It appears that there is a low threshold focus about 0 mm

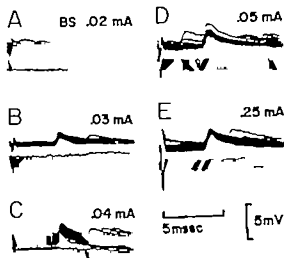


Fig. 2. Intracellular records (upper traces) from PBSt motoneurone on graded stimulation of the brain stem in the low threshold focus for the monosynaptic EPSP at the strengths indicated. 0.02 mA (A) was just threshold strength for the monosynaptic EPSP. Lower traces are recorded from the L7 dorsal root entry zone.

from the midline ipsilateral to the motoneurone and 2 mm below the bottom of the fourth ventricle. Fig 4 gives an idea of the effect of current spread around the electrode. The brain stem was stimulated at 7 times threshold for the monosynaptic EPSP When the electrod was moved from 2 to 5 mm below the bottom of the fourth entricle this stimulation did not evoke any monosynaptic EPSP The small EPSP in D was evoked with a segmental latency of 1.3 msec which suggests a disynaptic linkage. Fig 5 show threshold distribution for the monosynaptic EPSP when the electrode is moved in rostrocaudal and lateral directions. When the electrode is moved to midpontine and contralateral regions there is a considerable increase in threshold. It is therefore postulated that the pathway with monosynaptic connection with flexor motoneurones does originat from the upper medullary or lower pontine brain stem. The striking coincidence of the location of the low thresh-

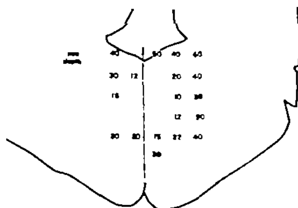


Fig. 3 Threshold distribution for the monosynaptic EPSP in PBSt motoneurone obtained from 5 different tracks in the brain stem. Each figure gives the multiples of lowest threshold (=0.01 mA) at position of the figure in plane 6 mm rostral to obex. The right side is ipsilateral to the motoneurone. The figures to the left indicate distances in mm.

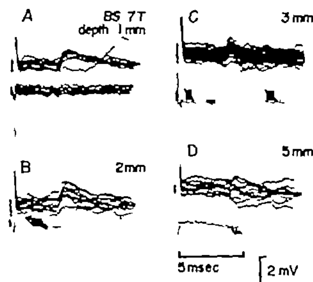


Fig. 4 Intracellula records (upper traces) from PBSt motoneurone The brain stem is stimulated at 7 times threshold for the monosynaptic EPSP t the same location as I Fig 1 at the dif ferent depths indicated below the bottom of the fourth entricle Lowermost trace in A show the corresponding extracellular field potential.



Fig 5 Threshold distribution as in Fig 3 but for horizontal plane th ough the brai stem, parallel to the bottom of the fourth entricle Each figure denotes the lowest threshold obtained one track, loc ted at the position of the figure Figures are mult ples of the lowest threshold for the monosynaptic EPSP which in all cases as 0.01 mA. The result were obtained from In PBSt motoneu ones in t experiment The scale to the left gives distances to obex in mm. The same scale is rue for ransverse distances. The dashed line indic tes the midline The right side is ipsilateral to the motoneurones

old focu w th the course of the ipsilateral medial longitudinal fascicle (iMLF and the narrow current range f r saturation of the EPSP makes it likely that the effects are evoked by tim lation of fibres rather than cell bodies in t[illegible] threshold focus. It is concluded from these experiments that the descending EPSP is mediated by fibres in the iMLF

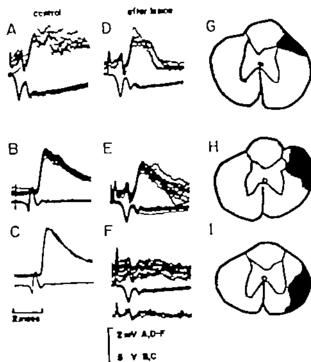


Fig. 6 Spinal location of the descending pathway with monosynaptic effects to PBSt motoneurone. The coVLF was transected and the dorsal columns removed for two segments at lower thoracic region (hatched area in G-I) and stimulating electrodes placed on the IVLF rostral to this lesion. During supramaximal stimulation of the IVLF (A, D—F) three lesions (black area in G-I) were made in the IVLF caudal to the stimulating electrode at different levels to permit histological control of each lesion. A shows the effect before any lesion of the IVLF. The record in D was obtained after the lesion (black area) in G E after the lesion in H and F after the lesion in I Record B and C show homonymous group Ia EPSP recorded after E and F respectively Lowermost trace in F show the extracellular field potential obtained after record C. Note that the intra- and extracellular traces in F are almost identical. Hence the initial part of the late positive potential in the intracellular traces in F is not an EPSP but an extracellular field potential.

The following spinal cord lesions were performed in one animal to obtain information about the location of the pathway with monosynaptic connections to lumbar flexor motoneurones. During intracellular recording from a PBSt motoneurone (Fig 6) successively deeper lesions were made in the ipsilateral lateral and ventral funicle (IVLF) at the lower thoracic level. Records D and E show that the monosynaptic EPSP is not changed after the lesion of the IVLF in G and H (black area) However after the lesion in I (black area) the monosynaptic EPSP is abolished (F) The records of the homonymous group Ia EPSP in B and C taken before and after lesion I show that the recording condition was not changed by the lesion which abolished the descending monosynaptic EPSP It is therefore concluded that the descending monosynaptic EPSP is evoked by impulses in ipsilateral fibres in the ventral part of the lateral funicle and the lateral part of the ventral funicle.

In Fig 6 it should further be noted that stimulation of the intact VLF evokes a polysynaptic EPSP in the control record A The dorsal lesion in G abolished most of this EPSP but revealed an IPSP in D In record E after the lesion in H, also this IPSP has disappeared and a pure EPSP remains. Hence it is suggested that the polysynaptic PSPs in A and D are not evoked by the pathway mediating the monosynaptic EPSP

2. *Extensor motoneurones*

Lund and Pompeiano (1968) demonstrated that the motor nuclei of some extensor muscles (for example G-S) receive monosynaptic EPSPs exclusively from the vestibulospinal tract which originates from the ipsilateral Deiters nucleus. However findings were presented suggesting that motoneurones of some extensors (for example ABSm) may receive a monosynaptic effect from a descending pathway originating outside the ipsilateral Deiters nucleus. In the present experiments monosynaptic EPSPs were evoked from the medial brain stem region in some G-S and ABSm motoneurones (Table 1) Fig 7 B shows the effect evoked in a G-S motoneurones from the lower thoracic region with a largely monosynaptic EPSP C-E are records obtained on increasing stimulation of the brain stem region described in section 1 It can be seen that in C no early postsynaptic effect is evoked The threshold for the monosynaptic EPSP is reached in D but the EPSP grows larger in E by the addition of component appearing 0.3 msec later than the low threshold EPSP This short delay cannot be caused by an additional synapse but it is not known if the delay is caused by activation of neurones with slower conducting axons or by activation of a more remote site of neurones with axons of similar conduction velocities. The relatively strong current required to evoke a maximal descending EPSP in Fig 7 suggests current spread (observe the large descending volley evoked by the stimulus ving the maximal EPSP) By contrast the EPSP in flexor motoneurones was urated within a much smaller current range However in some G-S motoneurones stimulation of the brain stem region described in section 1 evoked monosynaptic EPSP at the same low stimulus strength as for flexors and the effect was saturated within the same range of stimulus strength as for the corresponding effect in flexor

TABLE 1 Monosynaptic EPSPs (ME) evoked in three different species of motoneurones on stimulation of the ipsilateral VLF at Th 10 and the ipsilateral lower brain stem (BS) at the low threshold focus for ME in flexor motoneurones. First vertical row gives number of motoneurones showing ME_{VLF} The second row denotes number of motoneurones with threshold T for the ME_{BS} lower than the current strengths indicated. The third row indicates number of motoneurones where the ME_{BS} at 3 times T or less was of the same amplitude as the ME_{VLF} at supramaximal stimulus strength.

ME_{VLF}	ME_{BS}	$ME_{BS} = ME_{VLF}$
PBSt 29	T < 10 mA 28	22 (22 tested)
	T < 78 mA 29	
ABSm 1	T < 10 mA 9	7 (10 tested)
	T < 1 mA 10	
G-S 19	T < 10 mA 8	17 tested
	T < .35 mA 18	
	T < 80 mA 19	

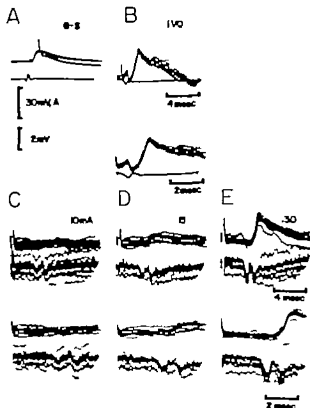


Fig 7 Intracellular records (upper traces) from G-S motoneurone. A shows a group I EPSP and the antidromic invasion on stimulation of the G-S nerve. B show the effect of supramaximal stimulation of the IVQ at the lower thoracic level. I C—E the brain stem is stimulated at the strengths indicated in the low threshold focus for monosynaptic EPSP in PBSt motoneurones. Upper and lower records in B—E were obtained simultaneously t two different time bases. Lower traces in each record are recorded from the L7 dorsal root entry zone.

motoneurones. A comparison of the monosynaptic effect to PBSt, G-S and ABSm motoneurones is given in Tabl I It ppears that in 6 of 19 G-S cells the descending EPSP is evoked at the same low strength that almost invariably (28/29) produces a monosynaptic EPSP in PBSt motoneurones. In all PBSt motoneurones tested (22) a stimulus strength of three times threshold evokes a monosynaptic EPSP of the same amplitude as that evoked from the spinal cord. Only in 2 of the 17 G-S motoneurones tested an EPSP of th same amplitude as that from the thoracic cord could be evoked within the same current range. Table I f rther shows that ABSm motoneurones w th respect to the threshold for th monosynaptic EPSP were more like PBSt than G-S motoneurones A moderat increase of the stimulus strength gave a maximal descending EPSP (i.e the same as from th thoracic cord) in 7 of 10 ABSm motoneurones. No threshold mapping has been performed to find th low threshold focus in the medial brain stem for these monosynaptic effects to extensors.

Stimulation of the medial brain tem region evoked IPSPs in several G-S motoneurones (Fig 8 C) This effect was lic ted t a stimulus strength within th weak range that gav monosynaptic EPSP n flex mot neurones. Hence there is the possibility that the same neuronal system ma evok reciprocal ffects excitation to flexor and inhibition to extensor mot neur nes

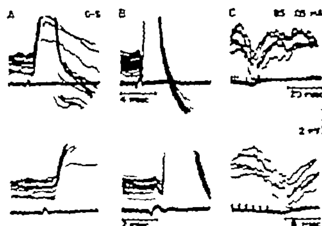


Fig. 8. Intracellular record (upper traces) from a G-S moto-neurone. A shows a group Ia EPSP and B the antidromic invasion on stimulation of the G-S nerv. C shows the effect of a short train of stimulus pulses at the strength indicated applied to the brain stem region from which low threshold EPSPs were evoked in PBSt motoneurones. Upper and lower records are obtained simultaneously at different sweep speeds.

Discussion

Threshold mapping in the brain stem has been employed to find the origin for the descending pathways with monosynaptic excitatory connection to flexor motoneurones. The results strongly suggest that the cells of origin are not located rostrally to lower pontine levels. Hence the rubrospinal tract cannot be responsible for the effect. Furthermore it was transected at the spinal level in some experiments by a lesion in the ipsilateral dorsal quadrant. It has also been shown that volleys in rubrospinal ...hways do not evoke monos naptic EPSPs in lumbar motoneurones (Hongo et al ...3). Since our finding suggests that within the medulla the fibres with this action descend in the MLF all the different s stems known to descend in the MLF will now be scrutinized as major candidates for the pathways with monos naptic effect on lumbar flexor motoneurones. The ipsilateral medial vestibular nucleus can be excluded since monos naptic EPSP could still be evoked in lumbar flexor motoneurones from the lower thoracic level several weeks after a lesion involving the ent re ipsilateral vestibular complex (Lund and Pompeiano 1968). Tectospinal fibres do not project to levels caudal to cervical segments (Nyberg Hansen 1964a). Furthermore these fibres originate from a region rostral to the low threshold focus obtained in the present stud and thus can be excluded on both anatomical and physiological evidence. Fibres from th interstitial nucleus of Cajal project to lumbar level (Nyberg Hansen 1966) but also this nucleus is situated rostal to the low threshold f us and apparentl few degenerated fibres of large caliber can be found following le on in the interstitial nucleus of Cajal (Nyberg Hansen 1966) while the descend ng fibres w th monos naptic onnecti ns to lumbar flexor motoneurones conduct t high velocity. Henc it is not likel that th interst tial nucleus of Cajal is the supraspinal origin for these effec. There is anatomical evidence for a contralateral vestibulospinal pathwa cros g t brain tem level nd descending in the MLF (see Pompeiano and Brodal 1 ; Nyberg Hansen 1964b). The higher threshold obtained from the contralateral side in th present results uggest that the pathwa is uncrossed in the medulla. If so this w uld exclude that the descending fibres evok ng monosynaptic EPSP in lumbar flex r mo oneurones originat from the contralateral

vestibular nuclei. Experiments with chronic lesions of this crossed vestibulospinal pathway are probably necessary entirely to exclude this possibility. Finally, a lesion abolishing the descending monosynaptic EPSPs in PBSt motoneurones leaves the medial part of the ventral funicle intact (Fig. 6). Since the pathways so far discussed, including a crossed bulbospinal component (see below), occupies this region (Busch 1961) none of them is likely to be responsible for the effect.

The medullary and lower pontine reticulospinal pathways will now be considered. Both pathways project to lumbar levels but only very few fibres enter lamina IX of Rexed (1954) where the motoneurones are located (Nyberg-Hansen 1965). The dendritic arborization can, however, be extensive (Cajal 1911, Lorente de Nó 1938, Aitken and Bridger 1961, Sprague and Ha 1964) and thus the synapses from descending fibres can be located on the dendrites. The pontine reticulospinal tract descends in the ventral funicle, the medullary reticulospinal in the lateral funicle, but there is not a complete separation (Torvik and Brodal 1957). Since the spinal cord lesion abolishing the monosynaptic EPSP in PBSt motoneurones (Fig. 6) occupies a border region in which any one of these two pathways may descend this experiment cannot be used to differentiate effects evoked from the pontine and medullary reticulospinal tract. Pontine reticulospinal fibres originate mainly from the nucleus reticularis pontis caudalis, while medullary reticospinal fibres originate mainly from the nucleus reticularis gigantocellularis (Torvik and Brodal 1957). The former pathway is uncrossed while the latter to some extent is crossed (Torvik and Brodal 1957, Busch 1961). Both pathways originate from cells of all sizes (Torvik and Brodal 1957). Within the brain stem the pontine reticulospinal fibres and crossed medullary reticulospinal fibres descend in the MLF but the uncrossed medullary reticulospinal tract descends in more lateral parts of the lower brain stem (Busch 1961). By analogy with the crossed vestibulospinal pathway, the crossed component of the medullary reticulospinal pathway also seems less likely to be responsible for the effect. If the anatomical data are considered in relation to the present results it is likely that the pathway evoking monosynaptic EPSPs in lumbar flexor motoneurones originates from the ipsilateral nucleus reticularis pontis caudalis.

Lund and Pompeiano (1968) concluded that the ipsilateral Deiters nucleus is the only supraspinal origin for descending pathways evoking monosynaptic EPSPs to some species of extensor α-motoneurones, for instance those belonging to the G-S motor nuclei. It was therefore an interesting finding in the present experiments that monosynaptic EPSPs could be evoked in some G-S motoneurones at the same low threshold stimulus strengths as for the corresponding effects in PBSt motoneurones. With this low threshold it is unlik[illegible] that the [illegible]ffects [illegible] G-S motoneurones are due to spread of current to th[illegible] Deiters nu[illegible] or the lateral [illegible]estibulospinal tract. One possibility is that stimulation of th[illegible] from the vestibulospinal tract (Carpenter 1960). S[illegible] has been obtained in a further analysis (Grillner [illegible] 19[illegible]). Th[illegible] possibility that the effect in G-S motoneurones should be [illegible]ked [illegible] nal fibres descendin[illegible] in the MLF is contradicted both by an[illegible] physiological evidence. These fibres

originate in the medial vestibular nucleus (Nyberg Hansen 1964b) and an ipsilateral chronic lesion of this nucleus does not interfere with the descending pathway evoking monosynaptic EPSPs in G-S motoneurones (Lund and Pompeiano 1968) Also further evidence is gi en by the finding that monosynaptic EPSPs can still be evoked from the Deiters nucleus in G-S motoneurones after a lesion of the MLF (Grillner *et al* to be published)

Stimulation of the medial region evoked monosynaptic EPSPs at low threshold more frequently in ABSm than in G-S motoneurones (Table I) Lund and Pompeiano (1968) have already given evidence that ABSm motoneurones may receive monosynaptic EPSPs from a pathway originating outside the ipsilateral Deiters nucleus, and in a further analysis, in which both the Deiters nucleus and the MLF were stimulated it will be shown that the majority of ABSm motoneurones, and motoneurones belonging to the extensor muscles flexor digitorum longus et hallucis longus receive their monosynaptic EPSP from the MLF and not from the ipsilateral Deiters nucleus (Grillner *et al* to be published)

References

AITKEN, J T and J E BRIDGER. Neuron size and neuron population density in the lumbosacral region of the cat spinal cord *J A at (Lond.)* 1961 95 38—53

BUSCH, H. F M *Anatomical analysis of the white matter in the brain stem of the cat* Van Gorcum & Comp. N V Assen, 1961 Thesis

CARPENTER, M B Fiber projections from the descending and lateral vestibular nuclei in the cat *Amer J A at* 1960. *107* 1—22.

GRILLNER, S H HONGO and S L UND Descending pathway with monosynaptic action on motoneurones *A t physiol sc d* 1966 *68* Suppl 77 p 60

GRILLNER, S and S LUND A descending pathway with monosynaptic action on flexor motoneurones *E perie tia* (Basel 1966. *22* 390

HONGO T E J OWSK and A LUNDBERG. Effects evoked from the rubrospinal tract in *E perie tia* (Basel) 1965 *21* 525

LLOYD, D P C. Activity neurons of the bulbospinal correlation system. *J N rophysiol* 1941 *4* 115—134

LORENTE DE NÓ Synaptic stimulation of motoneurons as local process *J N rophysiol* 1938 *1* 195—206

LUND, S and O POMPEIANO Monosynaptic excitation of alpha motoneurones from supraspinal tructures in the *A t physiol scand* 1968 *73* 1—21

MAGNI, F and W D W Identification of reticular formation neurons by intracellular recording *A h ital Biol* 1963 *101* 681—702

NYBERG-HANSEN R The location and termination of tectospinal fibers in the cat. *E p N ol* 1964a. *9* 212—227

NYBERG-HANSEN R Origin and termination of fibers from the vestibular nuclei descending in the medial longitudinal fasciculus An experimental study with silver impregnation methods in the cat *J comp Neurol* 1964b *12* 355—367

NYBERG-HANSEN R Sites and mode of termination of reticulospinal fibers in the cat An experimental study with silver impregnation methods *J comp Neurol* 1965 *124* 71—99

NYBERG-HANSEN R Sites of termination of vestibulospinal fibers in the cat An experimental study with silver impregnation methods *Arch ital Biol* 1964 *102* 98—111

POMPEIANO O and A BRODAL The origin of vestibulospinal fibres in the cat *A h ital Biol* 1957 *95* 166—195

REXED B A cytoarchitectonic atlas of the spinal cord in the cat *J comp Neurol* 1954 *100* 297—380

SPRAGUE, J M and H H The terminal fields of dorsal horn fibers in the lumbosacral spinal cord of the cat and the dendritic organization of the motor nuclei *Prog Brain R Organization of the spinal cord* eds J C Eccles and J P Schade Amsterdam, Elsevier Publ. Co., 1964 *11* 120—154

TORVIK, A. and A. BRODAL The origin of reticulospinal fibers in the cat An experimental study *A at R c* 1957 *128* 113—138

WILLIS, W D J C WILLIS and W M THOMPSON Synaptic actions of fibers in the ventral spinal cord upon lumbosacral motoneurons *J N rophysiol* 1967 *30* 382—397

Acta physiol. scand. 1968. 74. 285—300

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Fusimotor Effects on Position and Velocity Sensitivity of Spindle Endings in the External Intercostal Muscle of the Cat

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Abstract

ANDERSSON, B. F., G. LENNERSTRAND and U. THODEN. *Fusimotor effects on position and velocity sensitivity of spindle endings in the external intercostal muscle of the cat.* Acta physiol. scand. 1968. 74. 285—300.

Position and velocity responses of spindle endings in the external intercostal muscle of the cat have been determined in the absence and in the presence of fusimotor fibre stimulation at constant rate. The length input signal had triangular wave form. A close functional resemblance was found between intercostal and leg muscle spindles: primary and secondary endings could be separated among intercostal afferents with the same methods that have been used in leg muscles; dynamic and static fibres were discerned and the fusimotor effects on position and velocity sensitivity were the same as in the hind limb; the ratio between dynamic and static fibres was about 1 : 3, i.e. the same as in the hind leg. Some dissimilarities existed: the number of intermediate endings was larger in the intercostal than in the leg muscles; position and velocity sensitivities were generally larger in intercostal endings. These discrepancies are discussed. As a general conclusion it is stated that a spindle model elaborated from studies on leg spindles can easily be modified to account also for intercostal spindle behaviour.

In the anesthetized or decerebrate experimental animal almost the only opportunity to analyze naturally-occurring movements are provided by the respiratory system. Work on respiratory muscles (Critchlow and Euler 1963, Eklund, Euler and Rutkowski 1964, Sears 1964*b*, Corda, Eklund and Euler 1965) lends support to the hypothesis of a 'follow up length' servo-mechanism over the fusimotor system and the muscle spindles operating in conjunction with a direct action on the α motoneurones in the execution of aimed muscle movements (Merton 1953, Hammond, Merton and Sutton 1956). In order to reach further understanding of the nature of the control functions of the spindle, it seemed advantageous to proceed by alternating physiological and model studies on a motor system which showed natural movements, e.g. the intercostal muscles.

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In the present study a comparison between intercostal and hind limb spindle properties was made in order to determine whether or not a spindle model elaborated from data on leg muscle spindles could be directly applied to the intercostal system.

Both primary and secondary endings in the intercostal muscles were examined with the same methods that were employed in our earlier studies of leg muscle spindles (Lennerstrand 1968*a*, *b*, Lennerstrand and Thoden 1968*b*, *c*).

Some preliminary results obtained with sinusoidal length changes have been reported at an earlier occa ion (Andersson and Lennerstrand 1966).

Methods

Gener l p oc d r The experiments were performed on 15 cats, weighing between 1.9 and 3.7 kg. The animals were anesthetized with pentobarbital (Nembutal 40 mg/kg b.w. Injected i.p.) The operatn procedures needed for the laminectomy in the thoracic segments 6 to 9 and for the ttachment of special aluminium clamp t the ert brae for fixation of the preparation, has been described in detail in earlier publications from this laboratory (*cf.* Critchlow and Euler 1963, Corda, Euler and Lennerstrand 1966). The nerves to the internal intercostal muscles were cut in the appropriat segments lose to the exit of the spinal nerves from the ertebral olumn. The ribs and the intercostal muscles of the 6th t 9th thoracic interspace were freed from overlying tissue.

The last operative steps were performed w th the cat suspended in box (see below). These teps ncluded isolation of the intercostal segment t be studied by sectioning the ribs somewhat dorsal t he osteo-c rtilagenou j nction and cutting through the muscles p t the ertebral lumn in the segments abov and below. Thereupon the tercostal segment (usually the 8th) was positioned in the myograph, to be described below.

B the surgery t isolat he segment, pneumothorax was induced. During the experiments animal ere artif ially entilated. To prevent deh dration and cooling through the wide opened hes the animal was placed tight box, i which the temperature co ld be kept t bod temperature by means of heating lements and the ai could be kept aturated w h moisture by intermittent spr ng of warm Ringer solution. (Despit these pre tion he in ercostal prepar tion deteriorated more rapidly than leg muscle preparation and no experime t lasted longer han for seven hours from the moment the intercostal seg ment w isola ed and posi ioned in the myograph.) The rtebral lamp was fixed to he roof of the bo Ac ess the spinal cord was given through hole the roof. After incision of the dur he al and dorsal root were cut lose t the cord. Manipulations with the muscle prepa n nd he myograph were made through door the front wall of the box. The rec l emper ture as ll as he temperature of the intercostal muscl and of the oil the pro ering he spinal ord was on rolled be between 36 and 38 C.

My g ph The des gn of the myograph made of bras is show schematically in F 1. It was screwed he roof of the box. The segment w th the ex ernal intercostal muscle be studied *M* m l l w attached t he myograph by lamping the ribs of he segment. The 'lem of the myogr ph ere adjusted to parallel the ribs as fa possible. Before isolating the segment from he hest w ll the position of the ri with respect the perpendicular was noted and the width of he segmen measured. hes cond tions were also restored as far as possible when posit oning he segment int he myogr ph.

M hanical st m lat Length hanges of trapezoidal was form, w th extension and relaxations of constan peed follow ng immedia h upon each other and of frequencies arying between 1.3 and p ere generated by motor-driven am as described in previous paper (Lennerstrand and Thoden 1968 The am acted on the myograph ex stiff brass bar. The myogr ph and the motor were lectrically isolated from each other. The deviations from the ide l w form ere he same as hose reported the experiments on hind-limb muscles, maximally % of the total amplitude. The amplitude of the movement was 1.6 mm which is about 1.1 of the d tanc between the ri he segment when me sured along the muscle fibres. Bec use of he small amplitude he loc ties of the hanges of length were considerably lower than hose pplied to hind-limb pindles. The maximal speed in he intercostal experiments was 6.4 mm/sec.

F meter f br stim latio Afferent fibres were isolated in plit en al root f laments in the same way a has been desc ibed ber for the l mbar region. However, in the hort

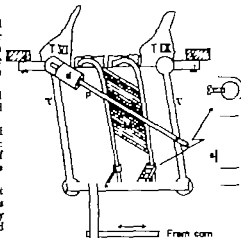


Fig. 1 Schematic diagram of the experimental arrangements The ribs in the segment under study articulate on the seventh and the eighth thoracic vertebrae. They are clamped to the myograph. The ribs and the 'legs' of the myograph (T) roughly parallel each other The length and the tension of the external intercostal muscle (M) can be measured simultaneously and independently of each other The length measuring device is differential transformer (d) with its iron core in plastic tube (p) Tension is recorded by means of semiconductor strain gauges ( ) glued to the steel blades of the hind rib clamp (see inset) Length changes are produced by cams (not shown) and transferred to the myograph as indicated in the bottom part (picture slightly modified from Andersson and Lennerstrand 1966)

ness of the ventral roots, it was found almost impossible to divide them into fine enough strands to get only one functionally isolated fusimotor fibre usually the ventral root filaments contained few α-fibres in addition to the activ fusimotor fibre( ) By tests in both the hind-limb and the intercostal preparations t was established that the unloading of the spindle in extrafusal contraction by co-activ ted α-fibres did not influence the position and locity sensitivities of the spindle endings, provided the tension dev loped by the -stimulation was low (below 10—15 g in the intercostal muscle, value which is about 5 % of the total tension that can be evolved) Of course, no exact measurements on the acceleration in the steady discharge by the fusimotor stimulation could be performed under these circumstances.

Trains of stimulus pulses were pplied to the entral root filaments t the fixed frequencies of 35 70 and 200 shocks/sec. Most of the results during fusimotor stimulation to be presented were obtained with rate of stimulation of 70 pulses/sec.

R cordings. Afferent muscle spindle activity was recorded and the impulse frequency of the discharge was calculated in the same way as described before (Lennerstrand and Thoden 1968a) Muscle length was monitored by the same differential transformer labelled *d* in Fig. 1 as was used in the hind-limb experiments The long axis of the transformer was approximately parallel to the muscle fibres as indicated in Fig. 1 which also shows the ttachment to the myograph of the transformer and of the plastic tube carrying the iron core Muscle tension was measured by means of semiconductor strain-gauges (Kyowa KGN-3.5-E2) labelled in Fig 1 The strain-gauges were glued to the lamp on the hind rib as shown in the inset of Fig 1 Recordings of electrical correlates to impulse frequency and muscle length and tension on time scale, or in plots of impulse frequency (*f*) against length (ΔL) were performed.

Proced re t determ e cond ctio elocity of afferent fibr The external intercostal nerv was stimulated between the tip of metal electrode and Ringer soaked sheet electrode of about 25 mm² area. The latter rested on the muscle tissue the metal electrode was inserted through the sheet electrode into the muscle near the nerv The nerv was stimulated in the dorsal part of the segment, before too extensiv branching had occurred. The conduction distance of the nerve between the stimulating metal lectrode and the recording electrode was in the order of 30 mm. The rate of nerv xcitation could not be exactly determined with this electrode arrangement. This showed p in the results as considerable ariation in the absolute values of conduction elocity (cf Fig 2 B) However the method was still useful as means of classifying spindle endings within single experiment.

Results

1 Classification of n -activated pi dl endings

Available anatomical (Barker 1962) and physiological evidence (Andersson and Lennerstrand 1966 Euler and Peretti 1966) indicates that intercostal spindles possess both primary and secondary endings

larger in length decrement than in length increment (Fig. 3 *B* and *C*). The $f/\Delta L$ diagrams depicted in Fig. 3 resemble closely those obtained from secondary, "intermediate" and primary endings, respectively, of the hind limb muscles (the intermediate endings were included in the group of primaries for reasons given by Lennerstrand (1968*a*)).

Thus, classifying the intercostal endings by means of the dynamic index would seem highly selective. Of the endings identified 17 fell into the group of secondary endings and 27 in the group of primary endings. Twelve of the primary endings showed responses of intermediate type at the lowest initial muscle extension applied. When the initial muscle extension was increased (maximally 0.8—1.0 mm) almost all primary endings exhibited $f/\Delta L$ characteristics of "intermediate" endings (*cf.* Lennerstrand 1968*a*).

2. Passive endings

Under the present experimental conditions, which with respect to muscle suspension and rib positioning were supposed to resemble quite closely those existing in the intact chest wall, all passive endings showed a position response (*i.e.* a steady impulse firing) even at the lowest initial muscle extension applied. At an arbitrary zero extension the secondary endings generally fired impulses at higher impulse frequencies (46 ± 17 S.D. imp/sec) than primary endings (23 ± 13 S.D. imp/sec).

Position and velocity sensitivities have been determined in the same manner as was described for spindle endings in the hind limb muscles (Lennerstrand and Thoden 1968*a*). Also in the intercostal spindle endings the velocity responses consisted of a quick and a slow part. They were assessed from the $f/\Delta L$ diagrams in the same way as was done for the leg muscle endings (see Lennerstrand 1968*a*). However, the variations in velocity responses within the groups of primary and secondary endings were generally larger in the intercostal than in the hind limb spindles, probably because of a smaller signal to noise ratio (impulse frequency/length) or to interspike interval scatter in the intercostal experiments. This in turn is likely to be due mainly to the small input signals.

The position sensitivity of the primary endings was 10.4 ± 5.5 S.D. imp/sec/mm. The position sensitivity of the secondary endings (5.9 ± 2.4 S.D. imp/sec/mm) was significantly lower. In the leg muscle spindles no significant difference was obtained between the position sensitivities of primary and secondary endings (Lennerstrand 1968*a*). An explanation for this discrepancy between intercostal and hind limb spindles cannot be given.

The velocity responses of the secondary endings investigated are collected in Fig. 4. In Fig. 5 the mean values obtained to length increment in the non-activated primary endings are represented by the broken lines. The vertical bars in both Fig. 4 and 5 mark the total range of the observed values. The input velocities are given in a log scale in the abscissa. The relations of the two components of the velocity response to the input velocity were similar in the intercostal and the leg muscle spindles. The quick velocity response of both secondary and primary endings and the slow

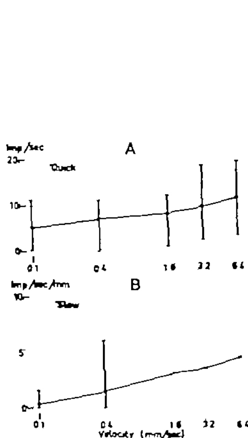


Fig. 4

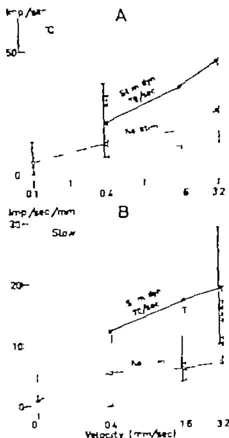


Fig 5

Fig 4 Primary secondary intercostal endings. *A* Average values of 'quick' component of velocity response and *B* of 'slow' component plotted against the input velocity on log scale. Vertical bars mark the total range of values in the samples.

Fig 5 Primary intercostal endings. *A* Average 'quick' velocity responses and *B* average 'slow' velocity responses against log input velocity Hatched lines represent non-activated endings and full lines endings during dynamic fusimotor activation at 70 pulses/sec Vertical bars as in Fig 4

velocity responses of secondary endings all increased with velocity over most of the range applied. In the upper velocity range the slow velocity responses of non-activated primary endings showed a very limited change (Fig 5 *B*) in resemblance to leg primaries. As to the absolute values of the two components of the velocity response the quick and the slow' component of the primaries and the slow' component of the secondaries were larger in the intercostal muscles than in the leg muscles at the same velocity sometimes twice as large. The quick component of the secondary endings was about the same in both types of muscles. These conditions were found to hold also for the velocity responses during fusimotor activation.

3 Endings under fusimotor activation

Repetitive stimulation of intercostal fusimotor fibres elicited either dynamic or static effects on the dynamic index corresponding to the observations on the hind limb spindles (Matthews 1962 Crowe and Matthews 1964*a* *b*) The identification of the static fusimotor fibres from their effects on the *f* ΔL curves as described in an earlier paper (Lennerstrand and Thoden 1968*c*) could easily be pursued in the intercostal experiments. Examples are given in Fig. 6 D and *E* of static fusimotor effects on a primary ending and in Fig 7 *B* and *D* of the same kind of fusimotor effects on a secondary ending

Dynamic fusimotor stimulation regularly caused the intercostal primaries to fire impulses during a large part of the length decrement. It was therefore difficult to know whether the fusimotor effect was evoked only by dynamic fibres or whether in most cases co-activation of dynamic and static fibres occurred (*cf* Lennerstrand 1968*b*) It should be recalled that in the thoracic ventral root filaments it was difficult to ascertain that single fusimotor fibres had been functionally isolated (see Methods) Acquaintance with unmingled dynamic effects in intercostal *f* ΔL diagrams was made by activating the spindles with succinylcholine. The most long-lasting part of the spindle activation induced by this drug is known to be of purely dynamic fusimotor type (Rack and Westbury 1966, Lennerstrand 1968*b*) Supplied with this experience interference in the dynamic fibre preparations of static fusimotor fibres could be recognized and avoided by further splitting of the ventral roots. In Fig 6 B and C a pure dynamic fibre preparation has been stimulated.

Dynamic fusimotor effects Dynamic fibres excited intercostal primary endings only Eight dynamic fusimotor fibres have been extensively studied. On tetanic stimulation their action was exactly the same as that observed in the hind limb (Lennerstrand and Thoden 1968*b*) *i e* to decrease the position sensitivity of the primary endings and to increase both the quick and the slow velocity responses to length increment. At a stimulus frequency of 70/sec the position sensitivity was decreased by 3.5 ± 4 3 S.D imp/sec/mm below the value in the non-activated state. The velocity responses during dynamic activation, shown in Fig 5 *A* and *B* were also obtained at a rate of stimulation of 70/sec. The slope of the curve relating the quick velocity response to the input velocity was increased by the dynamic fibre activation (Fig 5 *A*) During dynamic activation the slow component increased with increasing input velocity over a much larger range than in the non-activated state (Fig 5 *B*)

A levelling off or 'frequency ceiling' (Lennerstrand and Thoden 1968*b*) in the dynamic *f* ΔL curves to length increment was observed also in intercostal primaries subjected to high rates of dynamic fusimotor stimulation (Fig. 6 *C*) The relation between input velocity and the 'frequency ceiling' at a certain rate of stimulation was the same as in leg primaries, *i* linear in a log-log plot.

In their responses to length decrement during dynamic activation intercostal primary endings resemble more the intermediate endings of leg muscle than the typical primary endings (*cf* Lennerstrand and Thoden 1968*b*) Impulse firing was

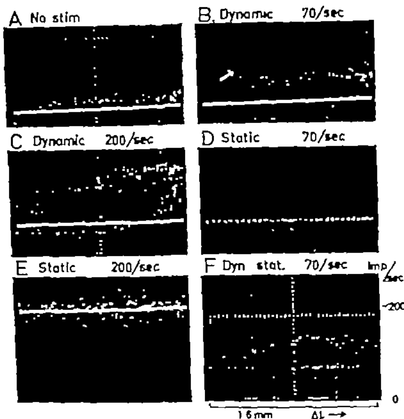


Fig. 6. *f*-ΔL diagrams of primary intercostal ending at 1.6 mm/sec 'triangular' length changes. *A*. Non-activated state. B and *C* dynamic fusimotor activation at 70 and 200 pulses/sec. *D* and E, static type I activation at the same rates. *F* dynamic-static co-activation at 70 pulses/sec. Markings as in Fig. 3. Note 'driving' in D and *F* steady state values coincide with driving' frequency

maintained during most of muscle shortening (*Fig 6 C*) A quick velocity response to length decrement could always be distinguished. It had the same time characteristics as the corresponding component of the velocity response to length increment. As in hind leg primaries, the total velocity response was smaller to length decrement than to length increment during dynamic activation (Fig 6 C)

Static fusimotor effect The influence of static fusimotor fibres can be studied in both primary and secondary intercostal endings During static fusimotor activation both types of ending fired impulses in length decrement along a curve parallel to that obtained in length increment, but shifted to lower values of impulse frequency (Fig. 6 *D* and *E* and 7 *B* and *D*) The velocity responses were regarded as being almost equal in length increment and decrement (*cf* Lennerstrand and Thoden 1968*c*)

In the hind limb spindles two kinds of static effect were recognized (Lenner

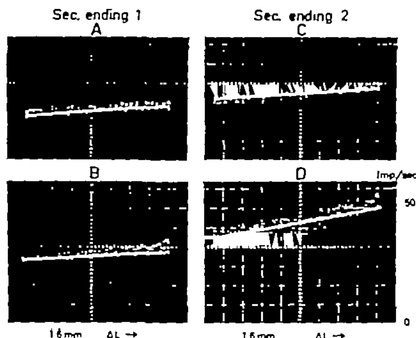


Fig. 7. f-ΔL diagrams of two secondary intercostal endings to 1.6 mm/sec constant velocity in triangular length changes. Non-activated state (*A* and *C*) and during 70 pulses/sec stimulation of two different static fibres (*B* and *D*). Type I static effect in *B* and type II action in *D*. Marking as in Fig. 3.

Strand and Thoden 1968c). Also in the intercostal muscles a corresponding subdivision of the static fibres could be made. Unchanged or reduced position sensitivity constituted the static type I effect, while the position sensitivity was augmented as a result of static fusimotor type II control. It should be observed that these functional groups of static fibres are not necessarily linked to certain anatomical nerve fibres: the same static fibre may have different effects in terms of type I and II on the various endings it innervates. Type I effects are shown in Fig. 6 *D* and *E* and in Fig. 7 *B*. Type II action is illustrated in Fig. 7 *D*. Seven type I effects and nine type II actions were observed in primary endings. In secondary endings type I effects were seen in six instances and type II effects nine times.

Driving of the afferent discharge could be observed in the f-ΔL diagrams only during static activation. This is exemplified by Fig. 6 *D*. Driving was seen in three cases of activating primary endings by route of fibres with type I action and at four instances when primary endings were under static fusimotor type II control. It was observed only once in a secondary ending when a fibre with type I effects was stimulated. Stimulations at 200 pulses/sec did not produce driving (Fig. 6 *E*). In the cases when driving occurred at all extensions in the f-ΔL diagrams, like in Fig. 6 *D*, the change in position sensitivity and the type of static fusimotor effect could be reliably determined only in the record obtained during stimulation at 200 pulses/

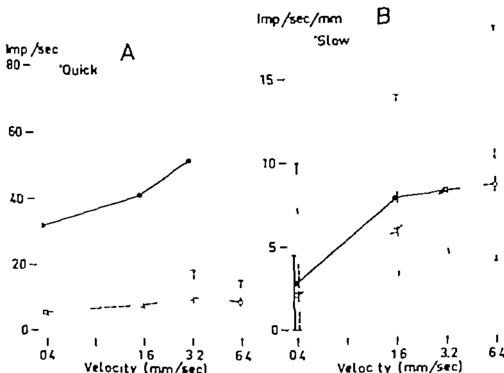


Fig. 8. V locity responses during static fusimotor activation of intercostal primary (full lines) and secondary endings (broken lines). A erage quick responses in *A* and 'slow' responses in *B* plotted against log input elocity. Vertical bars in Fig. 4.

sec. The changes in position sensitivity of stimulating static fibres at 200 shocks/sec are shown in T ble I.

With respect to the velocity responses recorded during static fibre stimulation no difference could be noticed between fibres of type I or II action. All velocity re sponses were of the common type of ankle spindl endings Lennerstrand and Thoden 1968*c*) During static activation of i tercostal spindles quick responses were larger in primaries than in secondaries (Fig 8 *A*) This is in ccordance w th findings in leg spindles. The static effects on the elocity esponses were th same in the intercostal and leg muscles. In secondary endings the quick response was decreased (the curve of Fig 4 *A* to be compared w th the hat hed curve of Fig. 8 *A*) and the slow response was increased (Fig 4 *B* to be compared w th the broken line in Fig. 8 *B*) Also in primary end ngs the slow response was ncreased t least i the upper elocity range (the curv of Fig 5 *B* to be compared with the solid curve in Fig 8 *B*) A direct comparison cannot be made between q ick responses of primary endings prior to and during static fibre timulation (*cf* Lennerstrand and Thoden 1968*c*)

TABLE I Cha ges in position sensitivity (from the alue in the non-activated stat) by stimulating static fibres with type I or II action t 200 pulses sec. Mea values nd standard deviations in impulses/sec/mm. Number of obser tions also given.

	Primaries		Secondaries	
	Mean ± S D	n	Mea ± S.D	
Type I activation	—5.6 ± 3.3	7	—1.2 ± 0.8	6
Type II activation	4 6 ± 3.5	9	6.5 ± 3.3	9

Endings activated by s veral fusimotor fibres Rather few experiments were made on the co-activation of two fusimotor fibre preparations influencing one spindle ending On the whole the results agreed with those obtained in hind limb spindles (Lennerstrand 1968*b*) For instance dynamic-static co-activation usually gave complete occlusion of the weakest single fusimotor effect in the position sensitivity and in the slope characteristics of the dynamic *f* ΔL diagrams. This is seen in Fig 6 *F* where static and dynamic co-activation at 70 pulses/sec was performed The individual effects are seen in Fig. 6 *B* and *D* respectively As for hind limb primaries, the mean impulse frequency to length increment d ring combined activation was usually shifted upwards to alues above those recorded during dynamic fibre stimulation alone

Discussion

The results indicate that there is a close functional resemblance between spindles in the intercostal nd in the ankle extensor and flexor muscles. For instance a subdivision of the elocity response into a quick and a slow component (Lennerstrand 1968*a*) could be performed also in the intercostal endings both in the absence and in the presence of fusimotor activation. Some similarities and disparities merit a closer examination

Interm diat ding In the group of primaries the so-called intermediat endings were more numerou in the intercostal than in the leg muscles. In the ankle extensors about 1/4 of the primary endings were of intermediate type while in the intercostal muscles about half of the primaries showed this type of response. Since the amount of initial extension on the pindle is known to determine the intermediate response pattern. firing of impulses during release of stretch (Lennerstrand 1968*a*) intercostal muscl would ppear to operate in a higher range of extensions than do the leg muscles This is indicated also by the fact that all endings fired impulses at the lowest initial extension when the muscle was suspended in the myograph.

Another way of explaining the larger number of intermediate endings in the intercostal material would be by assuming that the nuclear chain component of the

response of the primary ending (*cf* Lennerstrand 1968*a*) had a lower threshold of excitation in these muscles and therefore could contribute to the impulse firing during muscle shortening at a relatively low initial extension. All the intercostal endings, deprived of fusimotor activation, were active even at the lowest initial muscle extension when suspended in the myograph. It was therefore not possible to detect any difference between intercostal primaries and secondaries with regard to the threshold of muscle tension at which they started to fire impulses in the ankle extensor muscles, secondaries have higher threshold than their fellow primaries (Hunt 1954 Harvey and Matthews 1961) Actually the secondary endings of intercostal muscle generally had a higher rate of steady discharge than had the primary endings. This could be taken to mean that the secondaries and consequently the nuclear chain parts of the spindles, had the lowest threshold of excitation.

Structural differences between the intercostal and the leg muscle spindles might also be the basis for the relative abundance of intermediate endings in intercostal muscles. Intermediate forms of intrafusal fibres (Barker 1962) which could be proposed to yield intermediate types of responses, may be more numerous in intercostal spindles than in leg muscle spindles. Other possibilities are that the relative lengths of the nuclear bag and chain fibres or the number of intrafusal fibres in each spindle may be different in the two kinds of muscles, which, in some way or another might affect the thresholds to impulse firing. In this context it should be noted that both types of intrafusal muscle fibre are of about the same length in the spindles of the short interosseous muscles. These spindles contain a larger number of nuclear chain fibres than other leg muscle spindles (Boyd 1962) Whether this holds also for the short intercostal muscles is not known. These matters cannot be settled until the intercostal spindles has been re-examined with modern histological technique. A thorough histological investigation of these spindles and their afferent and efferent innervation seems a matter of high priority also from the fact that so much important work on the principles of motor control has been made on the respiratory system (see review by Euler 1966*a* *b* Sears 1966 and the monograph by Schwieler 1968) The significance of these results would increase still more if they could be correlated with intercostal spindle structure to the same extent as it has been possible in the hind limb muscles

Response values The mean values of position and velocity sensitivities were found to be higher for intercostal than for ankle extensor and flexor endings. The difference existed in the non-activated state as well as during fusimotor activation. (The position sensitivity and the quick velocity responses of the secondary endings constituted unaccountable exceptions from this rule being about the same in both types of muscl) These differences might depend on variations in the relative extensions applied to the spindles in the two groups of muscles in the ankle muscles the ratio of spindle length to muscle length is about 1 5—10 whereas this ratio in intercostal muscles is supposed to be in the order of 1 3—5 Accordingly an extension of the muscle with an amplitude of about 10 of the total muscle length will cause a larger relative extension of intercostal spindles than of ankle spindles and

give rise to seemingly larger position sensitivity in the intercostal endings. Also the rate of change of length will be relatively higher for intercostal spindles which therefore may appear to have larger sensitivities also to velocity. Certainly this point is of great functional significance because the same relative change of muscle length would be able to cause quite different amounts of afferent outflow from spindles in different muscles depending on the relation between the length of the muscle and that of its spindles. In case of intercostal muscles it is of importance to note that the amplitude of the extensions employed in these experiments (1.6 mm) are well within the limits of intercostal muscle excursions during quiet respiration (1—2 mm, Andersson and Lennerstrand, unpublished observation; see also records published by Critchlow and Euler 1963 and Euler and Peretti 1966).

Fusimotor activation. A distinct separation of the fusimotor effects into dynamic and static fusimotor actions could be made also in the intercostal spindles. As in the hind limb muscles these actions were identified from the change in dynamic indices of the endings induced by tetanic stimulation of functionally isolated fusimotor fibres in ventral root filaments (Matthews 1962, Crowe and Matthews 1964*a*, *b*, Appelberg, Bessou and Laporte 1966, Brown, Engberg and Matthews 1967) and from the f/AL diagrams to triangular length changes (Lennerstrand and Thoden 1968*b*). Because of technical difficulties (see Methods) it could not be definitely certified that only *one* fusimotor fibre to the ending under study was activated by the electrical stimulation; the efferent fibre preparation might have been composed of more than one fibre of the same kind. On the other hand, as pointed out above, wanted co-activation of different types of fusimotor fibres most probably did not occur. The ratio of the number of static fusimotor effects to dynamic fusimotor effects on the primary endings in this material is very near 1 : 3, which is the ratio given for leg muscles of the cat (Crowe and Matthews 1964*b*, Brown, Crowe and Matthews 1965).

Type I and II static fusimotor effects on the position sensitivity were recognized also in intercostal endings. The two types were about equally common in both primary and secondary endings. This distribution corresponds closely to that of ankle flexor muscles (Lennerstrand and Thoden 1968). Explanations for the variation in the distribution ratios to endings in different muscles will be discussed at a later stage of this spindle investigation.

All these findings tend to indicate that the efferent control of the muscle spindle has mainly the same functional organization in intercostal and leg muscles.

In conclusion of the above discussion it seems as if the differences were rather small between intercostal spindles and ankle extensor and flexor spindles with regard to static and dynamic properties. Hence the material for the spindle model in an analogue simulation of the neural control circuits of respiratory movements may to a great extent be collected from experiments on hind limb spindles. The advantages of using the leg muscles are firstly that small fusimotor fibre preparations can be more easily obtained, secondly that a larger range of velocities can be applied and, thirdly that the possibilities of preserving the preparation in good condition are

greater which, in turn, means that a more extensive dynamic analysis can be performed on each spindle ending.

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References

ANDERSSON, B. and G. LENNERSTRAND, Dynamic analysis of muscle spindles. In *Muscular afferents and motor control. Nobel Symposium I*. Editor R. GRANIT. Almqvist & Wiksell, Stockholm 1966. pp. 107—113.

APPELBERG, B., P. BESSOU and Y. LAPORTE, Action of static and dynamic fusimotor fibres on secondary endings of cat spindles. *J. Physiol.* (Lond.) 1966. *185*. 160—171.

BARKER, D. The structure and distribution of muscle receptors. In *Symposium on muscle receptors*. Editor D. BARKER. Hong Kong Univ. Press, Hong Kong 1962. pp. 227—240.

BOYD, I. A., The structure and innervation of the nuclear bag muscle fibre system and the nuclear chain muscle fibre system in mammalian muscle spindles. *Phil. Trans. B* 1962. *245*. 81—136.

BROWN, M. C., A. CROWE and P. B. C. MATTHEWS, Observations on the fusimotor fibres of the tibialis posterior muscle of the cat. *J. Physiol.* (Lond.) 1965. *177*. 140—159.

BROWN, M. C., I. ENGBERG and P. B. C. MATTHEWS, Fusimotor stimulation and the dynamic sensitivity of the secondary ending of the muscle spindle. *J. Physiol.* (Lond.) 1967. *189*. 545—550.

CORDA, M., G. EKLUND and C. v. EULER, External intercostal and phrenic motor responses to changes in respiratory load. *Acta physiol. scand.* 1965. *63*. 391—400.

CORDA, M., C. v. EULER and G. LENNERSTRAND, Reflex and cerebellar influences on and on 'rhythmic' and 'tonic' γ activity in the intercostal muscle. *J. Physiol.* (Lond.) 1966. *184*. 898—923.

CRITCHLOW, V. and C. v. EULER, Intercostal muscle spindle activity and its γ-motor control. *J. Physiol.* (Lond.) 1963. *168*. 820—847.

CROWE, A. and P. B. C. MATTHEWS, The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles. *J. Physiol.* (Lond.) 1964a. *174*. 109—131.

CROWE, A. and P. B. C. MATTHEWS, Further studies of static and dynamic fusimotor fibres. *J. Physiol.* (Lond.) 1964b. *174*. 132—151.

EKLUND, G., C. v. EULER and S. RUTKOWSKI, Spontaneous and reflex activity of intercostal gamma motoneurons. *J. Physiol.* (Lond.) 1964. *171*. 139—163.

EULER, C. v. Proprioceptive control in respiration. In *Muscular afferents and motor control. Nobel Symposium I*. Editor R. GRANIT. Almqvist & Wiksell, Stockholm 1966a. pp. 197—207.

E[U]LER, C. v. The control of respiratory movement. In *Breathlessness*. Editors J. B. L. HOWELL and E. J. M. CAMPBELL. Blackwell Sci. Publ. Oxford 1966b. pp. 19—32.

EULER, C. v. and G. PERETTI, Dynamic and static contributions to the rhythmic γ-activation of primary and secondary spindle endings in external intercostal muscle. *J. Physiol.* (Lond.) 1966. *187*. 501—516.

HAMMOND, P. H., P. A. MERTON and G. G. SUTTON, Nervous gradation of muscular contraction. *Brit. med. Bull.* 1956. *12*. 214—218.

HARVEY, R. J. and P. B. C. MATTHEWS, The response of de-efferented muscle spindle endings in the cat's soleus to slow extension of the muscle. *J. Physiol.* (Lond.) 1961. *157*. 370—392.

H[U]NT, C. C. Relation of function to diameter in afferent fibres of muscle nerves. *J. Physiol.* (Lond.) 1954. *87*. 117—131.

JANSEN, J. K. S. and P. B. C. MATTHEWS, The central control of the dynamic response of muscle spindle receptors. *J. Physiol.* (Lond.) 1962. *161*. 357—378.

LENNERSTRAND, G. Position and velocity sensitivity of muscle spindles in the cat. I. Primary and secondary endings deprived of fusimotor activation. *Acta physiol. scand.* 1968a. *73*. 281—299.

LENNERSTRAND, G. Position and velocity sensitivity of muscle spindles in the cat. IV. Interaction between two fusimotor fibres converging to the same spindle ending. *Acta physiol. scand.* 1968b. *74*. 257—273.

Lennerstrand, G. and U. Thoden, Dynamic analysis of muscle spindle endings in the cat using length changes of different length-time relations. *Acta physiol. scand.* 1968*a*. *73*. 234—250.

Lennerstrand, G. and U. Thoden, Position and velocity sensitivity of muscle spindles in the cat. II. Dynamic fusimotor single-fibre activation of primary endings. *Acta physiol. scand.* 1968*b*. *74*. 16—29.

Lennerstrand, G. and U. Thoden, Position and velocity sensitivity of muscle spindles in the cat. III. Static fusimotor single-fibre activation of primary and secondary endings. *Acta physiol. scand.* 1968*c*. *74*. 30—49.

Matthews, P. B. C., The differentiation of two types of fusimotor fibre by their effects on the dynamic response of muscle spindle primary endings. *Quart. J. exp. Physiol.* 1962. *47*. 324—333.

Matthews, P. B. C., The response of de-efferented muscle spindle receptors to stretching at different velocities. *J. Physiol.* (Lond.) 1963. *168*. 660—678.

Merton, P. A., Speculations on the servo-control of movement. In *The spinal cord. Ciba Found. Symp.* Editor G. E. W. Wolstenholme. Churchill, London 1953. pp. 247—255.

Rack, P. M. H. and D. R. Westbury, The effects of suxamethonium and acetylcholine on the behaviour of cat muscle spindles during dynamic stretching, and during fusimotor stimulation. *J. Physiol.* (Lond.) 1966. *186*. 698—713.

Schwieler, G. H., Respiratory regulation during postnatal development in cats and rabbits and some of its morphological substrate. *Acta physiol. scand.* 1968. Suppl. 304.

Sears, T. A., The fibre calibre spectra of sensory and motor fibres in the intercostal nerves of the cat. *J. Physiol.* (Lond.) 1964*a*. *172*. 150—161.

Sears, T. A., Efferent discharges in alpha and fusimotor fibres of the intercostal nerves of the cat. *J. Physiol.* (Lond.) 1964*b*. *174*. 295—315.

Sears, T. A., The respiratory motoneurone: integration at spinal segmental level. In *Breathlessness.* Editors J. B. L. Howell and E. J. M. Campbell. Blackwell Sci. Publ. Oxford 1966. pp. 33—47.

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Response Characteristics of Muscle Spindle Endings at Constant Length to Variations in Fusimotor Activation

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Abstract

ANDERSSON, B. F., G. LENNERSTRAND and U. THODEN. Response characteristics of muscle spindle endings at constant length to variations in fusimotor activation. *Acta physiol. scand.* 1968. 74. 301—318.

The aim of the present investigation was to determine the transfer function between fusimotor stimulus rate and afferent impulse frequency of muscle spindles at constant length. Spindle endings in the external intercostal and in the ankle extensor and flexor muscles of the cat have been subjected to variations in fusimotor stimulation frequency. The variations had sinusoidal form and ranged in repetition rate from 1/4 to 1/12 c/s. Both single-fibre and double-fibre stimulations were studied. Linear static and dynamic input-output relations were found in the stimulus frequency range 60 to 180 pulses/sec. For all combinations of one or two fusimotor fibres to one spindle ending the transfer function had the same general form. The static sensitivities were largest for primary endings under static fusimotor control; about the same sensitivities were obtained in dynamic fusimotor activation of primary endings and in static activation of secondary endings. With regard to dynamic properties all combinations of fusimotor fibres and spindle endings were equivalent. This was further tested with step and triangular wave inputs. Explanations for this similarity are discussed.

It is now a well known fact that an activation of the fusimotor system is often found to vary in parallel with that of the skeletomotor or alpha system, for instance in various reflex movements induced by electrical stimulation of central or peripheral nervous structures (Hunt 1951, Eldred, Granit and Merton 1953, Granit and Kaada 1953; for further references see Jansen 1966) and in naturally occurring respiratory movements (Critchlow and Euler 1963, Eklund, Euler and Rutkowski 1964, Sears 1964). Recent work suggests that co-activation of fusimotor neurones may occur also in voluntary contractions in man (Vallbo 1967). In order to get a complete quantitative understanding of the functional significance of muscle spindle control it is therefore of primary importance to obtain information on the static and dynamic properties of spindle endings to variations in fusimotor-input, and not only to changes in length-input as pointed out by Lennerstrand and Thoden (1968a).

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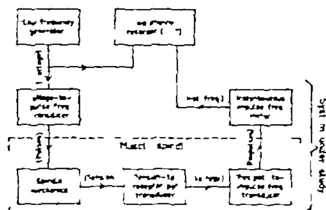


Fig. 1. Block diagram of the system under study and the stimulating and recording equipment.

The purpose of the present study is to analyse the static and dynamic responses of muscle spindle endings to defined changes in fusimotor activation while kept at a constant length. The response of primary and secondary endings of the ankle extensors and flexors and of the external intercostal muscles have been recorded during systematically modulated stimulation of dynamic and static fusimotor fibres.

In all earlier investigations touching this subject (e.g. Crowe and Matthews 1964) the only type of input signal employed has been step-like changes from zero in the rate of fusimotor fibre stimulation. Dynamic properties are hard to calculate with this input since the transients in the impulse frequency response to step input are of a relatively rapid time course and cannot be accurately resolved. Other ways of determining the spindle dynamics to varied fusimotor excitation were therefore attempted.

A convenient way to present the system under study is to represent it as a so-called block diagram, where the wave form generator, the voltage-to-frequency transducer, the intrafusal muscle fibres, the receptor zones, the receptor potential-to-frequency transducer and the instantaneous frequency meter each constitute one block (Fig. 1).

This diagram defines the model of the spindle, underlying the present investigation. The purpose of the investigation can now be stated more accurately: it is to describe the input-output relationship for as many of the blocks as possible. This description must take into account not only the static properties but also the dynamic ones. If the system is linear, and it will be shown below that it is in the stimulus range applied, the investigation of the system can be done conveniently by frequency analysis. This means that the input signal is varied sinusoidally and the amplitude and phase shift of the response relative to the input, is observed. The static response will be presented first. Thereupon the results from sinusoidally varying inputs will be described. Finally, as a test of the mathematical model derived from the sine fre-

quency analysis, results obtained with triangular wave and step inputs will be examined.

A preliminary report on some of the results has been published previously (Lennerstrand and Thoden 1967)

Methods

The experiments on spindles in the ankle extensor and flexor muscles were performed in 17 adult cats. Intercostal muscle spindles were studied in another group of experiments on 12 cats. The experimental procedures for examining hind leg muscle spindles were those described by Lennerstrand and Thoden (1968a) and the procedures for investigating intercostal spindles has been presented by Andersson, Lennerstrand and Thoden (1968) Identification of muscle spindle endings and of fusimotor fibres innervating them was performed from the spindle responses to 'triangular' movements as described in detail in the papers just quoted and in the papers by Lennerstrand (1968a) and Lennerstrand and Thoden (1968b)

In order to be able to subject single fusimotor fibres to varying stimulus frequencies, a low frequency generator (Hewlett Packard Model 202 A) was used which could deliver sinusoidal, triangular and step waveforms. This generator fed a voltage-to-frequency transducer with linear input-output relation, consisting essentially of a voltage-to-current transducer a capacitor and a uni-function transistor as discharging element. It was ended by a monostable flip-flop.

The range of the rates of stimulation could be chosen to lie anywhere between 20 and 400 pulses/sec. For the dynamic analysis mostly a range between 60 to 180 pulses/sec was used. Sometimes also variations between 60 and 120 pulses/sec were applied. In the frequency range from 20 to 400 pulses/sec the relative inaccuracy of the transducer was less than 5 % and in the range 60 to 180 pulses/sec it was less than % The periodicity of the triangular and sinusoidal variations were from 4 sec (in a few cases 8 sec) down to 1/12 sec (sometimes 1/16 sec) At periodicities longer than 8 sec intrafusal fatigue distorted the responses. At periodtimes shorter than 1/12 sec the measurements on the recordings became inaccurate because of the small ratio between the periodicity of stimulus variations and the afferent interspike interval. Constant rate stimulation of one fusimotor fibre while varying the stimulus rate of another fibre to the same ending was sometimes performed. The frequencies of steady stimulation were 70 and 200 pulses/sec The amplified spikes from the primary and the secondary endings were conventionally transformed into 'instantaneous impulse frequency (cf Lennerstrand and Thoden 1968a) and registered on photographic recording paper together with the input voltage to the voltage-to-frequency transducer

When measuring the static and dynamic properties of a system, it is always necessary to apply the input signal during a test period long enough to allow the transients in the response to vanish. On the other hand, in this special case too long stimulation could result in distortion of the response by intrafusal fatigue. As the measured time constants were in the order of 50 msec or less (see Results) all transients had subsided in a few hundred milliseconds. The static values of impulse firing were measured in the 2nd half-second period of constant rate stimulation (see also Lennerstrand and Thoden 1968b) When sinusoidal and triangular input signals were used it was sufficient to disregard the first two or three periods at the shortest period-times to get enough accuracy

The calculations necessary in connection with the different measurements are explained in detail below The accuracy of the results are difficult to estimate, even with statistical methods, due to the individuality of each spindle but as far as possible approximate values of the relative accuracies are given together with each type of measurement

Results

Static properties

Endings at constant extension. The steady state stimulus-response curves of both primary and secondary endings, at constant length to steady fusimotor activation by stimulation of either dynamic or static fibres were found to be S-shaped in the stimulus frequency range of 20 to 400 pulses/sec. In most endings there was an approximately

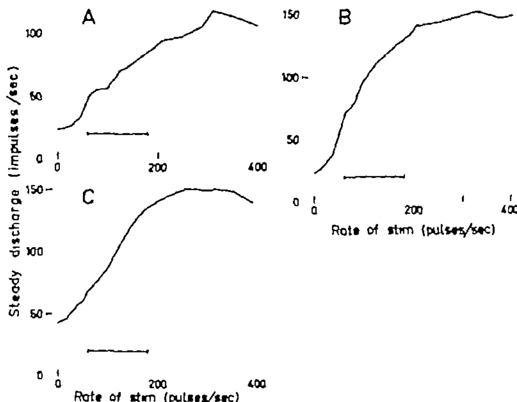


Fig. 2. Steady state curves relating rate of impulse discharge to frequency of fusimotor fibre stimulation. *A.* Extensor primary ending during dynamic fibre stimulation. *B.* Same primary ending during static fibre stimulation. *C.* Intercostal secondary ending during static fibre activation. The bars mark the range of stimulation rates between 60 and 180 pulses/sec.

rectilinear relation between the rate of stimulation and the impulse frequency of an ending within the stimulus range of 60 to 180 pulses/sec. This is exemplified in Fig. 2. Sometimes the linear relation was extended up to stimulus frequencies above 200/sec (Fig. 2 *A*). The curve approached its maximum value of impulse frequency at the stimulation rates of 250 to 300/sec. The latter finding is in accordance with the results of Hunt and Kuffler (1951), Eyzaguirre (1960), Harvey and Matthews (1961) and Bessou, Emonet Dénand and Laporte (1965).

In the present study the static sensitivity of an ending to varied fusimotor activation has been defined as the change in discharge rate caused by one unit of stimulus input in the rectilinear part of the static stimulus-response curve. Since the range 60 to 180 stimulus pulses/sec was the input amplitude most commonly applied in the dynamic tests (see below), it seemed practical to use this range as the 'unit' of stimulus input in the determination of static sensitivity. Average values of static sensitivity are given in Table I for all the endings studied. No significant differences were found between endings of the same type in ankle extensor or flexor muscles or in intercostal muscles. Primary endings activated by static fusimotor fibres of both

TABLE I Static sensitivity of primary and secondary endings to varied stimulation of dynamic or static fusimotor fibres. Values expressed as the increase in imp/sec by augmenting the rate of stimulation from 60 to 180 pulses/sec.

	Primaries		Secondaries
	Stim. 'dynamic'	Stim. 'static'	Stim. 'static'
Mean ± S.D	73 ± 23	124 ± 33	74 ± 37
number of obs.	12	16	13

type I and II (see Lennerstrand and Thoden 1968c) show the largest static sensitivities primary endings during dynamic fusimotor activation and secondary endings controlled by static fibres have about the same static sensitivities. This is in agreement with the findings of Appelberg, Bessou and Laporte (1966) who reported a larger increase in the steady discharge of primary endings during static fibre stimulation as opposed to activating dynamic fibres. On the other hand, Crowe and Matthews (1964) found about the same increase in the steady discharge rate of primary endings on stimulating both kinds of fusimotor fibres. This discrepancy between the results of different investigators must depend on the individual variations between spindle endings. The differences between the effects of static fibres on the steady discharge of primary and secondary endings seen in Table I are in accordance with the observations of Appelberg, Bessou and Laporte (1966)

Endings at increased muscle length The values listed in Table I were collected in muscle spindles at an arbitrary zero length. On extending the muscle spindle one would expect that the static sensitivity to fusimotor activation would increase when such fusimotor fibres were stimulated that increased the position sensitivity of an ending and *vice versa*. This was also found to be the case.

The muscle extension was in all instances increased by 8 mm. By this procedure the stimulus-response curve was shifted to higher ordinate values, but the curve was still rectilinear in the range of 60 to 180 stimulus pulses/sec. In 1[illegible] cases of stimulating a dynamic fibre to a primary ending the result was lowering of the static sensitivity to an average of 60 % of the sensitivity at zero extension (the range of the observed values was 40 to 79 %) The change in static sensitivity on stimulating static fibres with the spindle extended was about the same in primary and secondary endings. On activating static fibres of type I effect the static sensitivity was lowered to 88 % (n = 6 range of values 75—101 %) of the value at zero extension. Activation of static fibres type II resulted in an increase of static sensitivity to 122 (n = 10 range 110—138 %) of the original value.

Varied stimulation of two fusimotor fibres to the same ending Under natural conditions probably several fusimotor fibres to the same ending are concomitantly active When one dynamic and one static fibre to the same primary ending were

stimulated simultaneously with varying rates, the static sensitivity of the ending was maintained at the value of static single fibre stimulation in 5 out of 6 cases. In the remaining dynamic static combination a partial summation of the individual sensitivities was obtained. The results support the idea forwarded by Lennerstrand (1968*b*) that the dynamic and the static fibres might act on different intrafusal components and by means of complete occlusion the single fusimotor effect evoking the highest impulse discharge completely would dominate the spindle response in combined stimulation.

In other combinations, like *e.g.* two static fibres to a primary or a secondary ending, the fibres are likely to interact also by summation of mechanical and/or electrical events, as suggested from the effects on the spindle responses to changes of length (Lennerstrand 1968*b*). This view seems confirmed by observations on the static sensitivity in these fusimotor fibre combinations: the sensitivity could be either a partial summation of the sensitivities to single fibre stimulation, or take a value in between those of single fibre activation, or rarely coincide with the value of the single fibre giving rise to the largest static sensitivity.

Constant plus varied stimulation of two fusimotor fibres to the same ending. It was also tested how stimulation at a constant rate of one fusimotor fibre influenced the static sensitivity to varied stimulation of another fibre to the same ending, since this situation of "rhythmic" plus "tonic" fusimotor excitation occurs physiologically in respiratory muscles (Critchlow and Euler 1963, Eklund, Euler and Rutkowski 1964). In some cases of constant static fibre stimulation at 70 pulses/sec while varying the dynamic fibre activation to a primary ending in the usual 60 to 180/sec range, or at reversing the types of activation between the two fibres for that matter, no effect of the dynamic fibre stimulation could be seen in the impulse frequency response during combined activation of the ending. This could be explained in the same way as done earlier for the interaction between co-activated dynamic and static fibres. In other dynamic-static combinations some reduction of the static sensitivity to the varied activation was observed. On varied static fibre stimulation during constant static fusimotor activation of either primary or secondary endings, an increase in static sensitivity was sometimes seen. However, reversing the type of activation between the two particular fibres to the same ending — varied stimulation of the fibre which before was stimulated at a constant rate and *vice versa* — could now result in a reduction in static sensitivity as compared to the sensitivity to varied stimulation alone. Both the reduction and the augmentation of the static sensitivity was graded with the rate of the constant fusimotor stimulation. An unequivocal interpretation of these results is hard to reach at the present state of knowledge of spindle innervation and intrafusal fibre mechanics.

Dynamic properties

In order to determine the dynamic properties of the spindle endings, a sinusoidal voltage from the low frequency generator was fed to the system and the output from the instantaneous frequency meter was recorded. Results from the three different

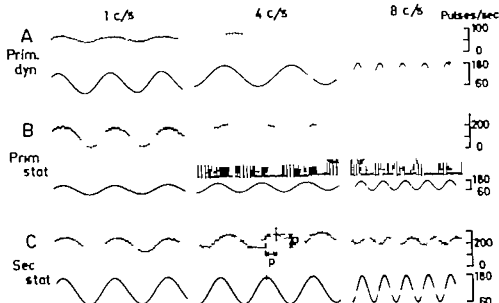


Fig 3 Impulse frequency responses (top record) to periodic fusimotor activation between 60 and 180 pulses/sec and of sinusoidal wave form (bottom record) Modulation frequencies 1 4 and 8 c/s. *A* Extensor primary ending: dynamic fibre activation. *B*. Another extensor primary ending: static fibre activation. "Scale of 2" circuit in use (compare position in time of action potentials at 4 and 8 c/s and the dots in the discharge rate record) Extensor secondary ending; static fibre activation. Measurements on response amplitude ( ) and phase lag relative input signal (p) shown in *C* (4 c/s) Records retouched.

combinations of fusimotor fibres and spindle endings are shown in Fig 3 *A B* and *C* for three different input signal frequencies. However before describing the results in more detail, it is necessary to determine if sinusoidal analysis gives meaningful results, *i.e.* if the system is linear It is also evident that the results must be presented in a more condensed form.

As pointed out above, the static relation between stimulus rate and instantaneous frequency output is linear in the stimulation range from 60 to 180 pulses/sec However to be allowed to apply linear analysis methods, usually sinusoidal analysis, it must be proved that the input to output relationship is linear in the entire dynamic range. The system is linear if it follows the so-called *superposition principle* In connection with sinusoidal analysis this means that if a sinusoidal input signal is fed into the system, giving rise to a sinusoidal output of certain amplitude and phase relative the input, and the input amplitude is then say doubled but the frequency unchanged) the output amplitude must also be doubled, but the phase remain the same This law must be valid for all input frequencies

The results from sinusoidal analysis can conveniently be presented in a so-called Bode plot (Fig 4) where the logarithmic ratio between output and input amplitudes and the phase relation between output and input signals respectively are presented as functions of the logarithm of the input signal frequency The amplitude ratio is given in dB and the phase shift in degrees. Normally the two functions represented in a Bode-plot can be summarized in one function of a complex frequency variable, called the *transfer function*. If the static curve has been shown to be linear and the input amplitude is kept constant it is convenient to compare the actual amplitude with the amplitude for very low frequencies (approximately static values) This means that the low frequency amplification is 1 (0 dB) for all investigated spindles and input amplitudes, thus making it easier to compare the results from different spindle endings.

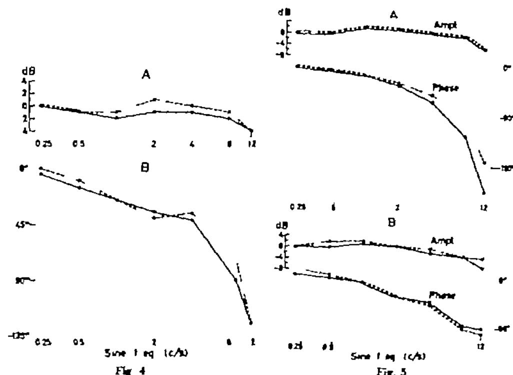


Fig. 4 Bode-plot. Extensor primary ending dynamic fibre activation. Amplitude change (*A*) in dB and phase shift (*B*) in degrees plotted against frequency of sine wave modulation of fusimotor activation. Full lines: fusimotor stimulation varies between 60 and 180 shocks/sec. Broken lines: between 60 and 120 shocks/sec

Fig. 5 Bode-plots *A* Extensor secondary ending static fusimotor activation at 60 to 180 pulses/sec Full lines fitted to values measured directly on original recordings (*cf* Fig 3) Broken lines after eliminating the influence of the voltage-to-frequency transducers. *B* Extensor secondary ending static fibre activation at 60 to 180 pulses/sec. Full lines ending at zero extension. Broken lines ending extended 8 mm. Note voltage-to-frequency transducers eliminated in this and in all the following Bode-plots.

Fig 4 shows the results from two sets of recordings, one where the input amplitude ranged from 60 to 120 pulses/sec and another where the input was doubled (60 to 180 pulses/sec)

Validity of the superposition principle implies identical Bode plots for both input amplitudes. As can be seen from Fig 4 the system is linear within the stimulation frequency range from 60 to 180 pulses/sec and within the tested range of repetition rates of sinusoidal input. The differences between the curves are within the error limits, which are provided below under Accuracy of the sine wave analysis Similar results were obtained also for other combinations of efferent and afferent fibres It seems therefore permissable to use linear analysis methods.

1 Sinusoidal wave input

Figures 5 and 6 illustrate typical Bode plots of a primary ending during sinusoidal dynamic fibre activation (Fig 6 *A*) of primary endings during static fibre activa-

tion (Fig. 6 *A* and *B*) and of secondary endings during static fibre activation (Fig. 5 *A* and *B*). The full lines in Fig. 5 *A* are fitted to values obtained directly in measurements from recordings like the ones shown in Fig. 3. The hatched lines in Fig. 5 *A* and all curves in Figs. 5 *B* and 6 connect values in which subtraction of the influence of the two voltage to-frequency transducers included in the system (*cf.* Fig. 1) has been performed. The latter procedure will be described below. It is observed that in all the combinations presented of a spindle ending activated by a single fusimotor fibre the phase and the amplitude characteristics are almost identical. This was a consistent finding: the differences between the individual Bode-plots of all endings investigated fell within the limits of errors given in a subsequent section.

It must be observed, however, that the Bode-plots include dynamic properties also for the outer voltage to-frequency transducer and the transducer from receptor potential to afferent impulse frequency. Very little is known about the latter transducer but we assume similar properties as for the outer transducer since this is indicated by results from studies on spindle responses to currents polarizing the sensory regions (Edwards 1955, Lippold, Nichols and Redfearn 1960). These properties together with an approximate Bode-plot for such a transducer are presented in Appendix.

Of the different blocks, shown in Fig. 1, we now know the transfer function for the voltage-to-frequency transducers (the last one ended with the instantaneous frequency meter) and the transfer function for the entire chain of blocks. The theory for linear system shows (see any ordinary textbook in control theory, *e.g.* Elgerd 1967) that one can obtain the transfer function for a chain of blocks connected in series from the transfer functions for the separate blocks by multiplying the individual amplitude ratios and adding the phase shifts. (As the amplitude ratios are expressed in dB one has simply to add these curves also.) To find the transfer function for a single unknown block in the chain one has to apply the reverse of these operations.

In Fig. 7 *A* the whole material on hind leg spindles (27 combinations of a spindle ending and a single fusimotor fibre) has been collected in a common Bode-plot and in Fig. 7 *B* the intercostal material (12 combinations) is presented. The influence of the two voltage-to-frequency transducers has been eliminated from each single Bode plot. The shaded areas show where phase shift and amplitude ratio respectively are to be found for the largest part of the spindle endings studied. There is a tendency for a summit point to occur in the amplitude ratio between input and output at sine frequencies between 0.5 and 4 c/s. This is probably a consequence of the reduction in amplitude at the lowest sine frequencies due to intrafusal fibre fatigue during the long stimulations. In spite of a large overlap there appears to be a small difference between hind limb spindles and intercostal spindles in the phase shifts to sinusoidal inputs in the upper frequency range. We will return to this variation later on (see p. 11).

A relevant question is now if it is possible from the reasoning outlined above to further divide the obtained transfer function of the spindle from fusimotor input to receptor potential into functions of two or more components. Intuitively one re

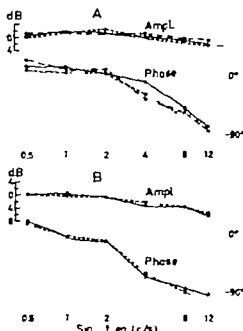


Fig 6

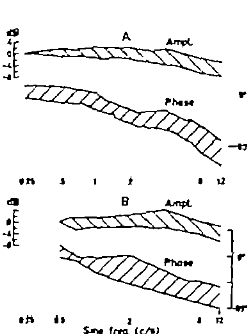


Fig 7

Fig. 6. Bode-plots *A* Extensor primary ending. Full lines dynamic fusimotor activation. Hatched lines static fusimotor activation. Pointed lines both fibres stimulated concomitantly All activations at 60 to 180 pulses/sec. *B* Extensor primary ending Full lines periodic static fibre activation 60 to 180 shocks/sec Broken lines to the periodic activation is added constant activation at 0 pulses/sec of another static fibre to the same ending

Bode-plots of 27 extensor endings fall within the shaded areas in *A* and those of 12 intercostal endings within the areas in *B* Fusimotor activation between 60 and 180 pulses/sec in all cases

that this can be done in a large number of ways. Taking into account simple facts about the internal structure of the spindle, it seems reasonable to assume that the spindle transfer function consists of that of a time lag and that of a dead time The time lag T is motivated by the assumption that after a suddenly applied fusimotor activation the tension acting on the receptor zones increases gradually due to the action of viscous elements in the intrafusal fibres. The dead time, τ is partly the time for impulse propagation in the efferent and afferent pathways and, partly the synaptic delays in the motor end plates and the sensory terminals on the intrafusal fibres. A Bode-plot for a time lag is shown in Fig. 8 A. One notices that the phase shift is limited to 90° which value tends to be exceeded in the spindle Bode-plots (Fig 7 The Bode-plot for a dead time is shown in Fig 8 *B* In this case there is a considerable phase lag but no damping of the amplitude ratio for higher frequencies, while damping of the amplitude was found for the spindle transfer function (Fig) However a suitable combination of a time lag and a dead time could account for the dynamic behaviour of spindle endings. By graphical methods presented in Appendix, values for T and τ can be found for the individual spindles. The results are given in

TABLE II Average values, calculated from Bode-plots, of the time lag, T and the dead time for spindle endings in extensor and intercostal muscles respectively. The values within brackets are the standard deviations for a single measurement.

	Extensors		Intercostals	
	T		T	
Mean ± S.D. (msec)	30 ± 3.5 (± 17)	11 ± 1 (± 4)	25 ± 1 7 (± 5)	10 ± 2 (± 6)
number of obs.	27	27	12	12

Table II. No significant differences were found between the different endings or fusimotor fibres in either hind leg or intercostal muscle. From the data available on conduction rates of the participating fibres one would expect differences of about 3—5 msec in dead times between spindles in ankle and intercostal muscles because of shorter nerve fibres from the spinal cord to the intercostal spindles. Such a difference, though suggested from Fig. 7 was not found, probably due to the rather poor resolution of the method used for the estimations.

Accuracy of the sine wave analysis. The signal-to-noise ratio was rather low especially for higher input sine frequencies, but not lower than 5 (cf. Fig. 3). The amount of distortion was not measured but it was tested that the error from distortion was in most cases negligible compared to that of noise. (A few units had considerable distortion and others stopped firing during part of each period; units with such behaviour were excluded.) The accuracy of an individual Bode-plot is thus in the order of ±2 dB for the amplitude ratio, maybe somewhat less for the lower sine frequencies and somewhat larger for higher frequencies, and in the order of ±10° for the phase shift at lower sine frequencies up to ±20° at higher frequencies. The rest of the areas in Fig. 7 are due to individual variations between different spindles.

The accuracy of the individual measurements of the time lag and the dead time is more difficult to estimate because it is necessary to combine them so that the two curves, the amplitude ratio and the phase shift, coincide. It is then rather difficult to avoid a certain amount of arbitrariness. Even without the dead time an estimation of the time lag would have been difficult because of the limited frequency range; experiments with higher frequencies should have been necessary but were not performable as pointed out in Methods. Thus, both the inaccuracy and the individual variations are included in the given standard deviations in Table II for the individual measurements.

Endings at extended length. Sinusoidal analysis was performed also on spindles at extended muscle lengths. The extensions were in all experiments 8 mm. Typical results from such experiments are shown in Fig. 5 *B* of a secondary ending activated by a static fusimotor fibre. As has already been pointed out under *Static properties* the static amplification was often changed by the muscle extension but no significant differences were observed in the Bode plots, e.g. in the dynamic properties relative to the results obtained at normal muscle length. It is thus concluded that, within the limits of error no changes in the dynamic properties were caused by muscle extension, at least as long as the extensions are within the physiological range.

Combined stimulation of two fusimotor fibres to the same ending. The dynamic properties as revealed by the Bode-plots were not changed significantly from those obtained in single fibre excitation when spindle endings were sinusoidally activated

by route of two fusimotor fibres. A typical example is presented in Fig 6 *A* However it should be observed that when a dynamic and a static fibre were concomitantly stimulated the static fusimotor effect usually completely dominated the summed impulse frequency response. Reasons for this behaviour have already been suggested. Furthermore, increased distortion was observed in some experiments when a dynamic-static fusimotor combination was stimulated.

Neither did constant activation of one fusimotor fibre and varied stimulation of another fibre to the same ending significantly change the spindle dynamics as indicated in Fig. 6 *B* Also in this type of double fibre activation of a dynamic-static combination the dynamic single fibre effect was usually 'occluded' by the static fibre stimulation.

Thus, double fibre activation of either of the kinds described, affected only the static properties of spindle endings to varied fusimotor excitation and not the dynamic behaviour of the endings.

2 Transfer function

After having divided the experimentally obtained transfer function of the spindle, described in the Bode plots into three factors, one depending on the voltage-to-frequency transformation, one on the dead time and one on the time lag we can now go the other way round and construct the Bode plot from these of the individual parts given in Appendix. The plots for the voltage-to-frequency transducer the time lag and the dead time are just added as explained above. The resultant transfer function of the spindle (input stimulation rate output afferent discharge rate) can be expressed mathematically in the following form (see Appendix)

$$K \quad e^{-s\tau} \quad \frac{1}{1+sT} \quad \frac{1-e^{-s\delta}}{s\delta}$$

gain factor	dead time	time lag	voltage-to-frequency transducer

where

- K static gain (from Table I)
- δ mean interspike time
- τ dead time (from Table II)
- T time lag (from Table II)
- s Laplace operator

With this transfer function the afferent discharge can be calculated for any time course of the variations in stimulation (see any textbook in transform calculus)

3 Triangular input signal

In order to test the result obtained with sinusoidal inputs and to get results with the same input signal as was used when the spindle endings were activated by mechanical

TABLE III. Comparison between the values of the quantity 0.69 T+ obtained by sinusoidal analysis (cf Table II) and by using triangular input waveform. The values of the standard deviation given within brackets are the deviations for single measurement. The results are for extensor muscles only

	Sinusoidal analysis	Triangular analysis
Mean value ± S.D (msec)	29 ± 3.5 (± 12)	27 ± 3 (± 10)
number of obs.	13	15

stimulation (Lennerstrand and Thoden 1968a) the spindles in the ankle muscles were also stimulated with triangular input signals (to the voltage to-frequency transducer) The time shift, Δ between the input voltage and the output signal was measured *at the moment when the input changed its direction from increasing to decreasing voltage* As usual, the output was obtained from the instantaneous frequency meter

As shown in Appendix the time shift can also be calculated from the results obtained with sine wave input.

Average values are shown in Table III Most of the individual results from the triangular tests were within the error limits obtained with sinusoidal inputs.

4 Step input signal

Measurements on the time lag were also performed with steplike input signals in order to test further the values obtained with sine wave input. The step change always originated from rates of stimulation above 40/sec. The values of T acquired with the step input were of the same order as those calculated from the Bode plots.

Discussion

The main new finding of this analysis of muscle spindle responses to defined changes of fusimotor activation is that the same linear transfer function applies to the dynamic input-output relation of all the possible combinations of one (or two) fusimotor fibres influencing one spindle ending The differences between the various groups of combinations with respect to dynamic properties fell within the error limits obtained in each group These results are rather remarkable in light of how dissimilarly the spindle endings reacted to mechanical stimulation during constant fusimotor fibre stimulation (Lennerstrand and Thoden 1968b If one makes the assumption that the receptor zones are sensitive to the tension in the intrafusal fibres, a possible explanation could be that no movements but only tension changes resulted from the varied fusimotor activation of the endings In this way the elements with viscoelastic properties in the nuclear bag and nuclear chain fibres respectively would have been largely by-passed If this is so the results also show that the dynamic properties of the receptor sites in nuclear bag and chain fibres are similar

The system studied, as shown in Fig. 1 could favourably be subdivided into components each contributing its share to the transfer function. The time constant of the part denoted spindle mechanics in the block diagram of Fig 1 has by indirect methods been calculated to be about 25—30 msec. The same value was obtained on stimulating the endings over both dynamic and static fibres. This value is considerably lower than that of 100 msec for step activation of primary endings by dynamic fibres indicated from experiments by Crowe and Matthews (1964 see also Smith, 1966) A time constant of about 100 msec would cause an amplitude reduction, relative to that obtained at slow frequencies of about 4 times or 12 dB at 6 c/s of sinusoidal modulation. Such reductions have never been observed in our experiments.

The only explanation for this discrepancy that we can find is that the earlier investigators have changed the *fusimotor stimulation in a step from no stimulation up* to a certain rate while we have always changed the fusimotor activation of the spindle ending from one state of excitation into another The latter method seems us to be more physiologically relevant.

The static gain factor assigned A in the transfer function, and termed static sensitivity was found to vary in magnitude with the kind of combination of spindle ending and fusimotor fibre. It would seem from these results as if the tension variations acting on the sensory terminals of spindle endings were the largest in case of static fusimotor activation of primary endings. There are reasons to believe that many static fibres mediate their effects over nuclear chain fibres in case of both primary and secondary endings (*cf* Lennerstrand and Thoden 1968*c*) One possible explanation for the differences in static sensitivity between primaries and secondaries during static fusimotor activation would then be that the receptor terminals of the secondary endings, which are located peripherally on contractile parts of the nuclear chain fibre, should be somewhat less loaded than the sensory terminals of the primary endings, which are situated centrally on largely non-contractile elements. The reason for the static sensitivity mediated by the dynamic fibres to primary endings being lower than that obtained in static activation of primaries may perhaps be differences in site of action on the ending of the two types of fusimotor fibre the dynamic fibres are thought to influence the nuclear bag fibres, while static fibres are believed to act also on nuclear chain fibres (*cf* Lennerstrand and Thoden 1968*b* *c*) and the two types of intrafusal fibre have been proposed to have different mechanical and contractile properties (Smith 1966)

Appendix

by

Bengt F. Andersson

I. Transfer functions for the components of the muscle spindle model

In the diagram of Fig. 1 the system under study is defined. The chain of blocks of the system starts and ends with voltage-to-frequency transducers. The input-output relations of the two blocks representing the spindle mechanics and the tension-to-receptor potential transducer have been further divided into a dead time and a time lag. In order to be able to determine these quantities it is thus necessary to know the transfer function for a time lag, a dead time and a voltage-to-frequency transducer.

The transfer function for a time lag is

$$\frac{1}{1+sT}$$

where

T is the time lag

s is the Laplace operator

The Bode plot for a certain value of the time lag, T, is given in Fig. 8 *A*.

The transfer function for a dead time is

$$e^{-s\tau}$$

where

τ is the dead time

s is the Laplace operator

One example is given as a Bode-plot in Fig. 8 *B*.

The transfer function for a voltage-to-frequency transducer is somewhat more complicated to find. The transducer is assumed to be of integral pulse frequency modulation type (cf. Jones, Li, Meyer and Pinter 1961). For very small relative variations of the input voltage the transducer will obviously be equivalent to a zero-order hold, the transfer function of which is

$$\frac{1-e^{-s\delta}}{s\,\delta}$$

where

δ is the average interspike interval

s is the Laplace operator

(cf. Bonvallion, Poppele and Terzuolo 1965). However, also for rather large relative variations of the input voltage this transfer function is a good approximation, provided that one measures the maximum variation of the output impulse frequency and that the input signal modulation frequency is reasonable much lower than the

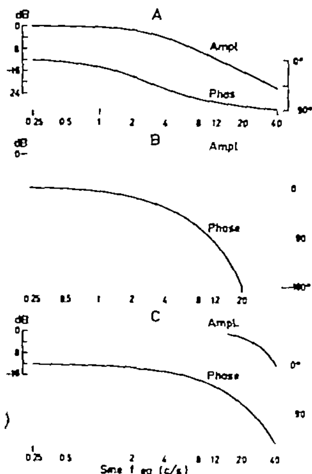


Fig. 8. A Bode-plot for time lag, *T*. The curves are drawn for *T* = 50 msec. *B* Bode plot for dead time. The curves are drawn for = 25 msec. *C* Bode-plot for the voltage-to-frequency transducer (approximative). The curves are drawn for value of the mean interspike interval of 20 msec. Note for other values of the constants the curves has the same shape as in *A*, *B* and *C* respectively but are shifted along the frequency axis.

output impulse frequency. For instance, if an input signal of low modulation frequency is applied, causing the output to vary sinusoidally between 30 and 90 imp/sec, the attenuation of this amplitude is predicted by the above given transfer function within one dB of the true value for signal frequencies up to 20 c/s. The phase shift is also well approximated if it is measured at the average interspike intervals. This example can be considered typical for the experimental results. An example of the above transfer function is given in Fig. 8 *C*.

When combining the different transfer functions into a model to fit the experimentally obtained results the following procedure was performed. The influence of the voltage-to-frequency transducers was first eliminated (subtracted) from the original plots. The resultant plot nearly always had some amplitude reduction with increasing frequency and the amplitude curve for the time lag was now fitted to the experimental curve. This normally gave the model smaller phase shift than the experimentally obtained. The dead time, causing phase shift only, was now fitted in to provide agreement between both amplitude and phase curves.

II Time sh ft between the input and the output of the system

Triangular input signals were used. The distortion of the signal in the subsequent blocks of the system is small and was neglected. In order to obtain the approximate total time shift between the maximal value of the triangular input and that of the impulse frequency curve to this input, the individual contributions of each block were added.

A triangular input to a time lag, T causes a time shift between the maxima of

$$T \ln 2 \cong 0.69 \; T$$

if

$$T \ll \text{input signal period time}$$

The solution can be found in *e.g.* Truxal (1955)

If the assumptions in Section I are valid, the voltage-to-frequency transducer has a transfer function of the type gi en above It can be shown, see *e.g.* Truxal (1955) that this transfer function causes a time shift between a triangular wa e and the response, of

$$\frac{\delta}{2}$$

where

δ is the average interspike interval,

That a dead time causes a time shift is trivial.

The resultant time shift, Δ is then

$$\Delta \cong T \; 0.7 + \sum \frac{\delta}{2} + \tau$$

where the summation sign indicates that both of th oltage-to-frequency transducers must be taken in account.

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References

Andersson, B. F G Len stra nd and L Thoden Fusimotor effects on position and velocity sensitivity of spindle endings in the external muscle of the cat *Act physiol. sc d* 1968. *74* 285—300.

Appelberg, B P Bessou and Y Laporte. Action of static and dynamic fusimotor fibres on secondary endings of cat spindles *J Physiol* (Lond 1966 *185* 160—171

Bessou P., F Emonet-Dénand and Y Laporte. Motor fibres innervating extrafusal and intrafusal fibres in the ca *J Physiol.* (Lond) 1965 *180* 649—67

Borsellino, A., R. E Poppele and C A Terz lo, Transfer function of the slowly adapting stretch receptor organ of Crustacea. *Cold Spr Harb Symp quan Biol* 1965 *30* 581—586.

Critchlow V and C E ra, Intercostal muscle spindle activity and its γ motor control. *J Physiol.* (Lond) 1963. *168* 820—84

CROWE, A. and P. B. C. MATTHEWS, The effects of stimulation of static and dynamic fusimotor fibres on the response to stretching of the primary endings of muscle spindles. *J. Physiol.* (Lond.) 1964 *174* 109—131

EDWARDS, C., Changes in the discharge from muscle spindle produced by electrotonus in the sensory nerve. *J. Physiol.* (Lond.) 1955. *127* 636—640

EKLUND, G., C. v. EULER and S. RUTKOWSKI. Spontaneous and reflex activity of intercostal gamma motoneurons. *J. Physiol.* (Lond.) 1964 *171* 139—163.

ELDRED, E., R. GRANIT and P. A. MERTON. Supraspinal control of the muscle spindles and its significance. *J. Physiol.* (Lond.) 1953. *122* 498—523

ELGERD, O. I. *Control systems theory.* McGraw Hill, New York 1967

EYZAGUIRRE, C. The electrical activity of mammalian intrafusal fibres. *J. Physiol.* (Lond.) 1960. *150* 169—185

GRANIT, R. and B. KAADA. Influence of stimulation of central nervous structures on muscle spindles in cat. *Acta physiol. scand.* 1953. *27* 130—160.

HARVEY, R. J. and P. B. C. MATTHEWS, The response of de-efferented muscle spindle endings in the cat's soleus to slow extension of the muscle. *J. Physiol.* (Lond.) 1961 *157* 370—3 .

HUNT, C. C., The reflex activity of mammalian small-nerve fibres. *J. Physiol.* (Lond.) 1951 *115* 456—46

HUNT, C. C. and S. W. KUFFLER, Further study of efferent small-nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* Lond. 1951 *113* 283—297

JANSEN, J. K. S. On fusimotor reflex activity. I. *Muscular afferents and motor control. Nobel Symposium I.* Editor R. Granit. Almqvist & Wiksell, Stockholm 1966, pp. 91—105

JONES, R. W., C. C. LI, A. L. MEYER and R. B. PINTER, Pulse modulation in physiological systems phenomenological aspects. *IRE Transactions Bio-Med. Electronics* 1961 *8* 59—67

LENNERSTRAND, G. Position and velocity sensitivity of muscle spindles in the cat. I. Primary and secondary endings deprived of fusimotor activation. *Acta physiol. scand.* 1968a. *73* 281—299

LENNERSTRAND, G. Position and velocity sensitivity of muscle spindles in the cat. IV. Interaction between two fusimotor fibres converging on the same spindle ending. *Acta physiol. scand.* 1968b *74* 257— 73

LENNERSTRAND, G. and U. THODEN. Responses of primary muscle spindle endings to constant or changing muscle length to variations in fusimotor activation. *Experientia* (Basel) 1967 *23.* 396—397

LENNERSTRAND, G. and U. THODEN. Dynamic analysis of muscle spindle endings in the cat using length changes of different length-time relations. *Acta physiol. scand.* 1968a *73* 234— 50

LENNERSTRAND, G. and U. THODEN. Position and velocity sensitivity of muscle spindles in the cat. II. Dynamic fusimotor single fibre activation of primary endings. *Acta physiol. scand.* 1968b *74* 16—29

LENNERSTRAND, G. and U. THODEN. Position and velocity sensitivity of muscle spindles in the cat. III. Static fusimotor single-fibre activation of primary and secondary endings. *Acta physiol. scand.* 1968c *74* 30—49

LIPPOLD, O. C. J., J. G. NICHOLLS and J. W. T. REDFEARN. Electrical and mechanical factors in the adaptation of a mammalian muscle spindle. *J. Physiol.* Lond. 1960. *153* 209—217

SEARS, T. A. Efferent discharges in alpha and fusimotor fibres of the intercostal nerves of the cat. *J. Physiol.* Lond. 1964 *174* 295—315

SMITH, R. S. Properties of intrafusal muscle fibres. I. *Muscular afferents and motor control. Nobel Symposium I.* Editor R. Granit. Almqvist & Wiksell, Stockholm 1966. pp. —80.

TRUXAL, J. G. *Control system synthesis.* McGraw Hill, New York 1955

VALLBO, Å. B. Impulse patterns in muscle nerves during voluntary contraction in man. *Acta physiol. scand.* 196 *69* 123—124

Acta physiol. scand. 1968. 74. 319—330

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Effect of Direct Tetanization on Twitch Tension in Developing Cat Leg Muscles

By

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Abstract

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The effect of tetanization, using both indirect and direct stimulation, on the twitch response to post-tetanic single shocks and on the tension response to a second tetanization has been studied in the gastrocnemius, soleus and extensor carpi radialis longus (ECRL) muscles of kittens ranging in age from 3—140 days, and in adult cats. Direct stimulation was applied in completely curarized animals. Nembutal® anaesthesia was used.

Equal tension was found to be produced by a first tetanization and a second one, the latter elicited during the period of twitch potentiation following the first. The second tetanization was, however, found to give a steeper rise in tension than the first.

In each animal, the maximal post-tetanic potentiation (PTP) of twitch tension obtained was of about equal magnitude following tetanization by indirect and direct stimulation. In newborn kittens, practically no PTP occurred in the gastrocnemius, whereas in the soleus there was a PTP of about 50 %. With age, the PTP produced by direct stimulation was found to increase in the gastrocnemius and decrease in the soleus, as after stimulation through the motor nerve. The time course of the effects produced by the two types of stimulation was also similar. After tetanization by both types of stimulation, potentiated twitches showed a steeper rise in tension than pre-tetanic control twitches, and in the gastrocnemius they also showed a marked lengthening of half relaxation time. In the kitten soleus, little change took place in the latter. Non-potentiated twitches of adult cat soleus muscle showed shortening of contraction and half relaxation time.

It is concluded that the main mechanism of twitch potentiation in the neuromuscular system of kittens and cats is located within the muscle fibres.

It was found in a previous investigation that, when using indirect stimulation, there is a high post-tetanic twitch potentiation (PTP) in the soleus of young kittens, the potentiation decreasing with age (Nyström 1968 a). In the gastrocnemius, no or little PTP occurred in young kittens, but an increase was observed with age. Since the localization of the mechanism responsible for the PTP phenomenon is unknown (see Hughes 1958), the nerve fibres (Nyström 1968 b), motor nerve terminals (Nyström 1968 c), subneural apparatuses and the activity of end-plate cholinesterases were studied (Nyström 1968 d) in the soleus and gastrocnemius during postnatal development, to ascertain whether postnatal changes in these parts of the efferent link could give any explanation of the observations regarding PTP. It was found that in the

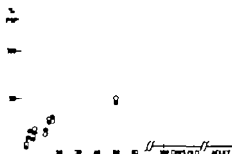


Fig 2 Maximal twit h potentiation in the gastrocnemius muscle of each animal (ordinate) using both indirect (open circles) and direct (filled circles) stimulation, is plotted again postnatal age (abscissa) Twitch potentiation is expressed in per cent of the pre-tetanic value of twitch tension. Tetanization for 9 sec t 45 or 100 c/sec, except in the adult cat (250 c/sec)

imposed tracings of tension records during two successive tetanizations in the gastrocnemius muscle of a 6-month-old cat, and Fig. 1 C is the same type of illustration, but in the soleus muscle of a 14-day-old kitten.

Similar results were obtained when the same type of experiment was carried out by using direct stimulation in completely curarized animals.

Twitch potentiation

Gastrocnemius muscle In Fig 2, the maximal PTP of twitch tension obtained in each animal after conditioning stimulation by indirect and direct 9 sec tetanizations of varying frequencies is plotted against postnatal age. As seen, the maximal PTP of twitch tension after tetanization by direct stimulation closely agreed with that recorded after tetanization by indirect stimulation in each of the young kittens, but at higher ages the variations were wider However also when using indirect stimulation ly ariations exist in the amount of PTP produced in successive trials (Nyström 1968 a)

The frequency-dependence of PTP following tetanization by direct stimulation appeared to be similar to that following tetanization by indirect stimulation (Nyström 1968 a) since, when using the former a first tetanization, if of high frequency elicited a lesser degree of PTP than a second one, if the latter was of low frequency When the frequencies were used in the inverse sequence—beginning with low-frequency tetanization—the twitch potentiation obtained after this first tetanization was always higher than that after a second one of high frequency The maximal post tetanic twitch potentiation found when using direct stimulation and plotted in Fig 2 was obtained after 9 sec tetanizations at 45 or 100 c/sec, except in the adult cat, where the frequency was 250 /sec, no lower one being tested.

The time course of twitch potentiation is seen in Fig 3 and in the records in Fig 4 A—B, from experiments on kittens 16 and 120 days old respectively As after tetanization by indirect stimulation (*cf* Nyström 1968 a) maximal twitch potentiation in young kittens was not achieved until 15—20 sec after cessation of the tetanization. With increasing age, the maximum was reached sooner after the end, and in old kittens and adult cats the first twitch (2.5 sec after cessation of the tetanization) already showed maximal PTP as also pplied when using indirect stimulation.

Twitch potentiation following tetanization by direct stimulation (Fig. 3) was

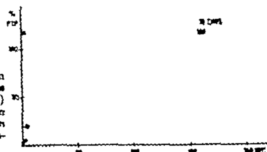


Fig. 5 Time course of twitch potentiation in the gastrocnemius muscle of tw kittens 16 (filled circles) and 120 (filled squares) days old. Direct stimulation. Tetanization for 9 sec at 45 c/sec Twitch potentiation (ordinate) plotted against time after cessation of the tetanization (abscissa)

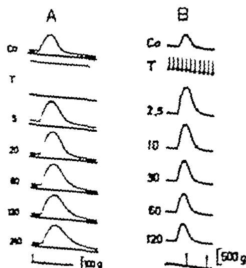


Fig 4 Twitch potentiation in the gastocnemius muscle of a 16-day-old (Fig. 3 A) and 120-day-old (Fig. 3 B) kitten after 9 sec tetanization at 45 c/sec. Direct stimulation. The time course is also seen in Fig. 5. Co denotes pre tetanic control twitch. T denotes tetanization, and the numerals time in sec after cessation of the tetanization. Time marking every 100 msec.

found to last for 6—8 min, thus about the same as that previously found after tetanization with indirect stimulation (6—10 min)

After the first and second tetanizations by direct stimulation—when deterioration of the preparation is still minimal—the effect of the conditioning tetanization on the contraction time of post-tetanic twitches can be seen In young kittens, showing little *PTP of twitch tension, a slight increase in contraction time of post tetanic twitches* sometimes occurred, although the changes were inappreciabl F g 3 A There was, however consistently a marked increase in half relaxa ion time post tetanically (Fig 5 A) Almost complete recovery to the pre teta ic ontrol alues oc rred after a period of rest (10—15 min) In old kittens Fig B nd d It is as well, no major change in contraction time but n nd ma ked ncrease in half relaxation time were recorded post tetanic B H however th lengthening of half relaxation time successively decreased d n g n test g period post tetanically as also found when using indirect stim l n 1968 a)

Soleus muscl The maximal PTP of tw h t d ned in the soleus muscl of each animal after 9 sec tetanizations of r eq encies, using both indirect

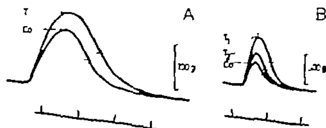


Fig 5 In A, two tracings from an experiment on the gastrocnemius muscle of 16-day-old kitten are superimposed, howing increase in half relaxation time of potentiated twit h (T) appearing 20 sec after essation of the tetanization, but no major hange in contraction time. Co denotes the pretetanic control twitch. Direct stimulation. Tetanization at 45 c/sec for 9 sec.
B is the same type of illustration but for 120-day-old kitten. Gastrocnemius muscle. Direct stimulation t 45 /sec for 9 sec. T1 and T are potentiated twitches ppearing 2.5 and 120 sec, respectively after cessation of the tetanization. Time 100 msec. See further text.

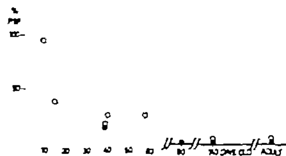


Fig 6 Maximal twitch potentiation in the soleus muscle (ordinate) of each animal using both indirect (open circles) and direct (filled circles) stimulation, plotted against postnatal age (abscissa) Twitch potentiation is expressed in per cent of the pre-tetanic value of twitch tension. Tetanization for 9 sec t 45 or 100 c/sec.

and direct stimulation, is plotted in Fig 6 against postnatal age. As seen in the figure and found previously (Nystrom 1968 a) the soleus muscle of young kittens showed a considerable twitch potentiation following tetanization through the motor nerve. A similar potentiation occurred after tetanization by direct stimulation as well. With age, the PTP produced gradually declined, and in the adult cat practically no twitch potentiation was elicited (*cf* Standaert 1964) As with the gastrocnemius muscle, low and moderate frequency tetanizations (45 and 100 c/sec) were more effective than high (250—450 c/sec) During the high frequency tetanizations—using direct stimulation—neither gastrocnemius nor soleus muscles were maximally contracted. They merely showed an initial contraction followed by a slow plastic yield, as also noted by Standaert (1964) All the values plotted in Fig 6 were recorded after direct tetanic stimulation at 45 or 100 c/sec

The time course and duration of the twitch potentiation seen in the soleus muscle of young kittens are shown by the records in Fig. 7 A and B comparing indirect and direct stimulation. In addition Fig 8 shows a plot of the post tetanic changes in twitch tension following tetanization by direct stimulation in two kittens, 10 and 140 days old, respectively As with indirect stimulation (*cf* Nyström 1968 a) maximal PTP of twitch tension in young kittens was reached 15—20 sec after cessation of the tetanization, as also applied to the gastrocnemius (*cf* Fig 5) The old kitten exhibited no twitch potentiation. The only change in size of the post tetanic twitches *found at that age occurred during the first 2.5—5 sec* following the tetanization, when the twitches were slightly reduced in size

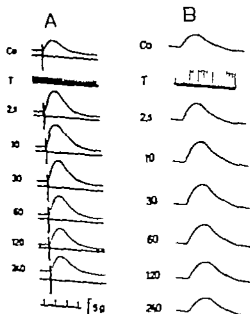


Fig. 7 Twitch potentiation in the soleus muscle of 10-day-old kitten, using both indirect (Fig 6 A) and direct (Fig. 6 B) stimulation. Co denotes the pre-tetanic control twitches. T denotes tetanization for 9 sec at 45 c/sec. The numerals indicate time in sec after cessation of the tetanization. Time marking every 100 msec.



Fig. 8. Time course of twitch potentiation in the soleus muscle of two kittens, 10 (circles) and 140 (squares) day old. Direct stimulation. Tetanization for 9 sec 45 c/sec. Twitch potentiation (ordinate) in per cent of pre-tetanic twitch tension plotted against time after cessation of the tetanization (abscissa).

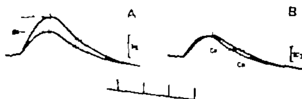


Fig 9 I A, two tracings from an experiment on the soleu muscle of 10-day-old ki ten are superimposed, showing slight increase in half relaxation time of potentiated twitch (T) ppearing 10 sec after cessation of the tetanization bu no hange in contraction time Co denotes the pre-tetanic control tw tch. Direct stimulation T anizatic 4 sec for 9 ec

B is the same type of illustration but for 14 -da -old ki en Soleu muscle. Direct stimulation at 43 c/sec for 9 sec. Co denotes be pre et tw and T post etanic twitch the appearing 2.5 sec af er cessation of the etaniza ion Not decrease in both cont acti half relaxation time post-tetanicall Time marking ev msec

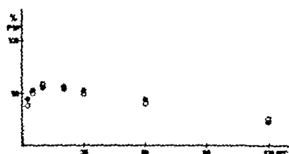


Fig 10. Time course of twitch potentiation in the ECRL muscle of a 10-day-old kitten, after indirect (open circles) and direct (filled circles) repetitive stimulation at 45 c/sec for 9 sec. Twitch potentiation plotted in per cent of pre-tetanic twitch tension (ordinate) against time after end of the tetanization (abscissa)

After tetanization by both direct and indirect stimulation (*cf* Nyström 1968 a) potentiated twitches in the soleus of young kittens showed no change in contraction time (Fig 9 A) as compared to pre tetanic twitches. When using direct stimulation, a lengthening of half relaxation time occurred (Fig 9 A) which was not consistently recorded when using indirect stimulation. As pointed out in Material and Methods, tetanization by direct stimulation seems to deteriorate the delicate kitten muscles (*cf* the aforegoing with respect to the gastrocnemius muscle) for which reason the evaluation of small changes in half relaxation time is difficult. In the soleus of old kittens (Fig 9 B) and the adult cat, a shortening of both contraction and half relaxation time took place post tetanically and lasted for about 1 min. This was also found when using indirect stimulation (Nyström 1968 a)

After a tetanization—irrespectively of whether direct or indirect stimulation was used—potentiated twitches in both the gastrocnemius and soleus showed a steeper rise in tension (Figs. 5 A—B 9 A)

Extensor carpi radialis muscle The changes taking place in this muscle following tetanization by direct stimulation were similar to those in the gastrocnemius muscle. The amount of PTP produced in young kittens was, however definitely higher in the ECRL (Fig 10) than in the gastrocnemius, as noted previously when using indirect stimulation. The time course and duration of twitch potentiation following tetanization by both direct and indirect stimulation can be seen in Fig 10 for the ECRL muscle. As can be inferred, close agreement was present between the effects following the two different ways of stimulation

Comments

In none of the three muscles investigated—gastrocnemius, soleus and ECRL—was the tension of single non-potentiated twitches higher when using direct stimulation than when using indirect. In contrast, the tension of directly induced as compared to indirectly induced twitches was often lower ranging from 0—11 % lower in the gastrocnemius, 0—32 % lower in the soleus and 0—10 % lower in the ECRL. The greatest reduction was noted in young kittens whereas in adult cats and old kittens no major difference was present between the tension produced by indirectly and directly induced twitches.

Repetitive potentials accompanying a single directly induced twitch were never recorded in any of the three muscles investigated.

Discussion

If in normal muscles, recruitment of muscle fibres does not take place during a tetanus but post tetanically as in curarized muscles (Liley and North 1953) there should be a post-tetanic increase in tension not only of single twitches but also of a tetanus. Since, however in the three muscles investigated the tension of two successive tetani was found to be equal (Fig 1) no post tetanic recruitment of muscle fibres apparently exists in normal muscle. The possibility still remains that a recruitment of fibres takes place already during the first tetanus, explaining why the two tetani have the same tension. It is known that, under certain conditions, the end-plate potentials may increase during repetitive stimulation (see Eccles 1964) In the curarized preparation, on the other hand they decrease (Liley and North 1953)

A close agreement is present between the results obtained after direct and indirect stimulation with respect to (a) developmental changes in magnitude of PTP in the muscles investigated, (b) the time course of PTP at various ages and (c) also with respect to contraction and half relaxation time of post as compared to pre tetanic twitches. This indicates that the results obtained are probably similarly generated—by some change in the contraction mechanism. This change can thus be brought about either by stimulation through the motor nerve or by direct stimulation. This indicates that a possible postnatal change in the events taking place post tetanically in the nerve, in its terminals or at the neuromuscular junction, are phenomena that have no relation to the postnatal change in PTP Furthermore, these phenomena must be of minor importance, if any for the generation of PTP of twitch tension (except in adult cat soleus muscle, *cf* Nyström 1968 a and d) The PTP induced by these mechanisms is probably due primarily to an increase in size of the end-plate potentials (Liley and North 1953 see also Nyström 1968 e) although Standaert (1963 1964) suggested that repetitive firing is generated within th motor nerve terminal itself.

Since twitch potentiation is considerably less marked after high frequency tetanization during which the muscle fails to contract maximally owing to Wedensky block (see Shamarina 1960) than after low frequency tetanization, it appears as if maximal contraction of the muscle fibres favours the appearance of post tetanic twitch potentiation. In partly curarized muscles which, on tetanization show only a brief initial contraction followed by quiescence during the rest of the tetanization period (Feng *et al* 1938) the PTP of twitch tension is very short, and is brought about by a mechanism other than that existing in normal, non-curarized muscles (Nyström 1968 e) In partly curarized muscles, twitches larger than those before curarization were never recorded during decurarization, which further supports the view that maximal contraction of the muscle fibres during tetanization is necessary for the generation of PTP of twitch tension.

Whether the PTP that is based on a mechanism located within the muscle fibre itself is brought about primarily by a change in the muscle fibre membrane or in the contractile mechanism *per* obviously not be determined from the present experiments. In the tibialis anterior muscle of the adult cat, Bowman, Goldberg and

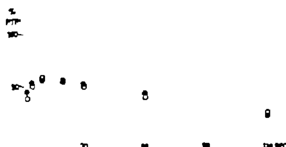


Fig. 10. Time course of twitch potentiation in the ECRL muscle of a 10-day-old kitten, after indirect (open circles) and direct (filled circles) repetitive stimulation at 45 c/sec for 9 sec. Twitch potentiation plotted I per cent of pre-tetanic twitch tension (ordinat) against time after end of the tetanization (abscissa)

After tetanization by both direct and indirect stimulation (*cf* Nyström 1968 a) potentuated twitches in the soleus of young kittens showed no change in contraction time (Fig 9 A) as compared to pre-tetanic twitches. When using direct stimulation, a lengthening of half relaxation time occurred (Fig. 9 A) which was not consistently recorded when using indirect stimulation. As pointed out in Material and Methods, tetanization by direct stimulation seems to deteriorate the delicate kitten muscles (*cf* the aforegoing with respect to the gastrocnemius muscle) for which reason the evaluation of small changes in half relaxation time is difficult. In the soleus of old kittens (Fig 9 B) and the adult cat, a shortening of both contraction and half relaxation time took place post tetanically and lasted for about 1 min. This was also found when using indirect stimulation (Nyström 1968 a)

After a tetanization—irrespectively of whether direct or indirect stimulation was used—potentiated twitches in both the gastrocnemius and soleus showed a steeper rise in tension (Figs 5 A—B, 9 A)

Extensor carpi radialis muscle The changes taking place in this muscle following tetanization by direct stimulation were similar to those in the gastrocnemius muscle The amount of PTP produced in young kittens was, however definitely higher in the ECRL (Fig. 10) than in the gastrocnemius, as noted previously when using indirect stimulation. The time course and duration of twitch potentiation following tetanization by both direct and indirect stimulation can be seen in Fig 10 for the ECRL muscl As can be inferred close agreement was present between the effects following the two different ways of timulation.

Comments

In none of the three muscles investigated—gastrocnemius, soleus and ECRL—was the tension of single non-potentiated twitches higher when using direct stimulation than when using indirect In contrast the tension of directly induced as compared to indirectly induced twitches was often lower ranging from 0—11 % lower in th gastrocnemius, 0—32 % lower in the soleus and 0—10 % lower in the ECRL. The greatest reduction was noted in young kittens whereas in adult cats and old kittens no major difference was present between th tension produced by indirectly and directly induced twitches.

Repetitive potentials accompanying a single directly ind ced twitch were never recorded in any of the three muscles investigated.

ever a consistent and marked increase in half relaxation time occurs post tetanically which is not the case in young kitten soleus muscle (*cf* Nyström 1968 a) Furthermore, potentiated twitches in the adult cat gastrocnemius are accompanied by muscle action potentials increased in duration, which does not apply to the kitten soleus (Nyström 1968 a) Because of this increase in duration, recruitment of fibres cannot be excluded as a cause of a minor part of the PTP found in normal gastrocnemius and ECRL muscles of the adult cat (Nyström 1968 a)

The soleus of young kittens is known to have a poorly developed sarcotubular system, less developed than that in the fast white tibialis anterior (Orkand 1964) Furthermore, young kitten soleus has a much higher tetanus/twitch ratio than fast white muscles (Buller and Lewis 1965 b, Close and Hoh 1967) In view of these observations, it is tempting to speculate that, in a soleus twitch of young kittens, some myofibrils or actin-myosin cross-bridges are unactivated unless the muscle is made to contract by tetanic stimulation, after which they are activated by some mechanism, thereby adding tension to the post tetanic twitch. The finding that partly curarized and normal soleus of young kittens showed equal changes with regard to contraction and half relaxation times, comparing pre and post tetanic twitches (Nyström 1968 e) is also compatible with the hypothesis of a recruitment of myofibrils or cross-bridges as the cause of PTP in the soleus muscle fibres of young kittens.

The most likely explanation of the reduction of twitch tension in directly elicited twitches, as compared to indirectly elicited ones, is destruction of some muscle fibres when sewing the stainless steel wire (stimulating electrode) in several loops through the muscles. In adult cat muscles, destruction of some fibres will be of little consequence for the total twitch tension. In the small kitten muscles, on the other hand—and especially the soleus, where sewing must necessarily be made through a large part of the muscle, due to its long bony origin from the tibia—destruction of small portions of muscle fibres will be of great consequence for the compound twitch tension.

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References

Bowman, W C A. A J Goldberg and C Raper A omparison between the effects of tetanus and the effects of sympathomimetic amines on fast and slow-contracting mammalian muscles. *Brit J Pharmacol.* 196 *19* 464—481
Brown, G. L. and U S von Euler, The after effects of etanus on mammalian muscle *J Physiol.* (Lond.) 1938. *93* 39—60
Buller, A. J and D M Lewis The rate of tension dev lopmen sometric tetanic contractions of mammalian fast and slow sk letal muscle *J Physiol* (Lond) 1965 *1 6* 337—354
Buller, A. J and D M. Lewis, Further observations on he differe iation of skeletal muscles in the kitten hind limb. *J Physiol.* (Lond. 196 b *17* 355—370
Close, R. and J F Y Hoh Force velocity prop rties of ki muscles. *J Physiol* (Lond 1967 *192* 815—822.

COLOMO F and P ROCCHI Staircase effect and post tetanic potentiation i frog nerv -single muscle fibre preparations. *A k. Fisiol.* 1965. *64* 189—266

ECCLES, J C., *Th physiology of synapse* Springer Verlag Berlin, Göttingen, Heidelberg 1964

ECCLES, J C. and C. S. SHERRINGTO Numbers and contraction-values of individual motor units examined in some muscles of the limb. *P oc roy Soc B.* 1930. *106* 326—357

EDMAN K. A. P and D W GRIEVE, The role of alcium and zinc i the electrical and mechanical responses of frog sartorius muscle. *Experientia* (Basel) 1961 *17* 557—558.

EDMAN, K. A. P and D W GRIEVE, On the role of calcium in the excitation-contraction process of frog sartorius muscle. *J Physiol.* (Lond.) 1964 *170.* 138—152.

FENG, T P., L. Y LEE, C. W MENG and S -C. WANG, Studies on the neuromuscular junction. IX. The after effects of tetanization on n—m transmission in cat. *Chin se J Physiol* 1938. *13* 79—108.

FENN, W O The fate of potassium liberated from muscles during activity *Amer J Physiol.* 1939 *127* 356—373

GAGE, P W and J I. H BRARD, An investigation of the post-tetanic potentiation of end-plate potentials at a mammalian neuromuscular junction. *J Physiol.* (Lond.) 1966. *184* 353—375

HILL, A. V The abrupt transition from rest to activity in muscle. *Proc roy Soc B.* 1949 *136* 399—420.

HILL, A. V The plateau of full activity during a muscle twitch. *Proc oy Soc B.* 1953 *141* 498—503.

HUGHES, J R. Post-tetanic potentiation. *Physiol. Rev* 1958. *38* 91—113

HUTTER, O. F Post-tetanic restoration of neuromuscular transmission blocked by d-T bo-curarine. *J Physiol* (Lond.) 1952. *118* 216—227

LILEY A. W An investigation of spontaneous acti ty at the neuromuscular junction of the rat. *J Physiol.* (Lond.) 1956. *132* 650—666.

LILEY A. W and K. A K. NORTH, An lectrical investigation of effects of repetitiv stimulation on mammalian neuromuscular junction. *J Neurophysiol.* 1953. *16* 509—527

NORRIS F H Active stat plateau and latency in mammalian striated muscle *Amer J Physiol* 1961 *200* 667—671

N STRÖM, B Mechanical and electrical responses to single shocks in developing cat leg muscles following tetanization. *Act physiol. scand* 1968 a. *74* *07—223

STRÖM B Fibre diameter increase in nerves to slow-red" and "fast-whit cat muscles during postnatal development *Act eurol sc nd* 1968 b. I prem.

NSTRÖM B Postnatal development of motor nerv terminals in "slow-red" and "fast-white cat muscles. *Act neurol scand* 1968 I press.

NYSTRÖM B. Histochemical studies on end-plate bound esterases in slow-red" and "fast white cat muscles during postnatal dev lopment. *Act eu ol. sc nd* 1968 d. I press

NYSTRÖM B Post-tetanic decurarization in developing cat leg muscles. *Act physiol. sc nd* 1968 e. *74* 226—237

ORKAND, P M Light and electron-microscopical studies of skeletal and cardiac muscle in the normal state, and in drug-induced myopathies in the cat Ph. D Thesis. Univ of London. 1964

RAMSEY R W and S F STREET Muscle f nction as studied in single muscle fibres. *Biol. Symp* 1941 *3* 9—34

RITCHIE, J M and D R WILKIE, The effect of previous stimulation on the activ state of muscle. *J Physiol* Lond) 1955 *130* 488—496

SANDOW A., S R T lor and H PREISER, Role of the action potential in excitation-contraction oupling *F d Proc* 1965 *24* 1116- 1123

SHAMARINA, N M Pessimal response of single muscle f bre to indirect stimulation *Sech nov physiol. J USSR* (Engl Transl 1961 *47* 1145—1153

S NDAERT F G Post-tetanic repetitiv activit in the cat soleus nerv *J gen. Physiol* 1963 *47* 53—70

STANDAERT F G The mechanisms of post-tetanic potentiation in cat soleus and gastrocnemius muscles. *J ge Physiol* 1964 *47* 987—1001

WALKER, S M The response of the triceps surae of normal, adrenalectomized, desoxycorticosterone acetate-treated and KCl-treated rats direct and indirect, single and repetitiv stimulation. *Amer J Physiol* 1947 *149* 7—23

WALKER, S M., Action potentials in ra muscle w th twitch tension potentiated by KCl treatment, drenalectomy tetanus nd treppe *Amer J Physiol* 1948. *154* 63—72.

WILANDER, B Activ stat d ration of ra gastrocnemius muscle *Act physiol scand* 1966. *68* 1—17

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Localization of Gastrin Activity in the Gastric Antrum[1]

By

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Abstract

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Gastrin activity was determined in sections, cut parallel to the surface, of the antral mucosa and submucosa of cats and dogs and in the aboral corpus mucosa of dogs and man. The maximal gastrin activity in the antrum was found in the basal 2/3 of the gland crypt region of the mucosa. The superficial 1/3 of the antral mucosa of the cat contained no gastrin activity whereas in the dog traces of activity were found in this part of the mucosa. Extracts from the submucosa of the cat antrum contained no gastrin activity but in the submucosa of 1 dog out of 3 low activity was found. The results suggest that the gastrin cells are localized predominantly in the region of the middle and basal parts of the gland crypts of the antral mucosa in the dog and cat. In the corpus mucosa immediately oral to the visualized antrum-corpus boundary no gastrin activity was found in 3 dogs and activity was present in only 4 of 14 duodenal ulcer patients. Thus the gastrin cells do not seem to be present in the corpus mucosa of dog or man, but the occasional finding of gastrin activity in the most aboral part of the acid secreting gastric mucosa of man speaks in favour of a dentate, or sometimes notchy antrum-corpus boundary.

The hormone gastrin has been recovered from the antral part of the stomach, and gastrin-like activity has been obtained also in extracts from the proximal duodenum, but never in extracts from the acid-secreting part of the stomach (see Emås and Fyrö 1968). The gastrin cell has not yet been identified, although it has been claimed that it might be a modified ganglion cell of the Meissner plexus in the submucosa (Baugh et al. 1958, Woodward et al. 1963). Recently an endocrine-like cell, displaying the morphological features of protein secretion, has been found in the mucosa of the antrum (Solcia, Vassallo and Sampietro 1967).

The gastrin cells may thus be distributed on both sides of the boundary between the antrum and the duodenum, but whether they also are to be found on both sides of the boundary between the antrum and the acid secreting part of the stomach is unknown. This question is not only of theoretical interest but has clinical signifi-

[1] A preliminary report of this study was presented at the Conference on Postgraduate Gastroenterology in Glasgow 1963 (Olbe and Elm[illegible]).
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cance as well since certain surgical procedures for the treatment of human peptic ulcer disease aim at removing most of the gastrin-releasing tissues.

In the present study extracts were prepared from sections of the mucosa and submucosa of cat and dog antra, cut parallel to the surface. These extracts were tested for gastrin activity in order to determine at which depth the gastrin activity was to be found. Furthermore extracts prepared from the gastric corpus mucosa immediately oral to the antrum-corpus boundary in dog and man were tested for gastrin activity.

Methods

Preparation of m sa pecimens. 25 cats and 4 mongrel dogs were used. The animals were anesthetized by i. injection of sodium pentobarbital (35 mg/kg) and the antra were removed. Each dog antrum was used separately while the cat antra were collected in 3 series, consisting of 5, 9 and 11 antra, respectively. The antral mucosa with the adhering part of the submucosa was separated from the muscular coat by dissection with blunt-edged scissors. Cylinders of tissue with a cross-sectional area of about 3 cm were cut out from the antral mucosa. The cylinders were immediately levelled against a plane surface frozen in isopentane t −70 C with liquid nitrogen, and stored t −20 C. Sections were cut perpendicular to the mucosal surface fixed and stained with toluidine blue for histological examination of the relationship between the thicknesses of the gland tissue and the submucosa in the cat and dog antral mucosa preparations. I each histological preparation the thicknesses of the gland tissue and the submucosa were measured t 3 predetermined points.

For determination of gastri activity at various depths, the frozen cylinders of antrum were cut parallel to the mucosal surface in cryostat at −30 C. The cylinders from the cat antra were cu into 40 slices, starting from the submucosa. Onl cylinders not differing by more than 80 μ in total thickness were used in each of the 3 series of cat antra. A fixed number of consecutive slices from every tissue cylinder in series were pooled for extraction of gastrin. 4 or 5 pools were collected in each of the at antra series Table I). Each pool thus represented definite layer in the antral mucosa.

The tissue cylinders from the dog antra were cu from and parallel to the mucosal surface in 40 or 35 μ dog D slices. This cutting proceeded for distance calculated to comprise the gland tissue. The calculation was based on the measurements in the histological sections cut perpendicular to the mucosal surfac The remaining tissu of the cylinders from each dog antrum was pooled. The approximate thickness of the uncut tissue was calculated from its weight by comparison with the weights of he cu slices. A fixed number of consecutiv h cut slices from all tissue cylinders of each dog antrum were pooled for extraction of gas in 3 to 7 pools each representing definite laye in the antral mucosa, were collected from every dog Table II I addition 10 section was taken at the end of each pool of slices from every cylinder for histological examination of the amoun of gland tissue t arious depths in the mucosal preparations Fre

Since part of the antral submucosa was attached to the muscular coat after the initial dissection procedur this part was stripped ogether with some muscular tissue from the antra of 3 ats and 3 dogs. This part of the submucosa was separately extracted for gastrin.

The aboral part of the corpus mucosa from 14 duodenal ulcer patients and 3 dogs w also extracted for gastrin. The boundary between the acid secreting and non acid secreting mucosa of the stomach was isualized during operation with pH indicator techniques. Olbe 1963, Berenström and Broomé 1964. This f nctional boundary was marked at several points with suture. The sutures were found to correspond to the histological boundary between the antrum and corpus in every ase. A 2 cm ide strip of orpus mucosa was taken immediately oral to the visualized antrum-corpus boundary. The strip was frozen for later extraction of gastrin. In two of the duodenal ulcer patients gastrin was also extracted from a cm wide strip taken immediately distal to the isualized antrum-corpus boundary.

Preparatio f gastr t acts. The mucosal preparations were weighed and minced. Gastrin extracts were prepared according to the method of Komarov 1938. All preparations from each human, dog or cat series were extracted simultaneously but separately. Care was taken to treat all batches identically.

Assay of gastrin activity. The acid secretory activity of each gastrin extract was assayed on non-anesthetized gastric fistula cat with histamine as the reference standard, as described by Uvnäs and Emås 1961. All extracts from ach series of at antra and from each dog antrum were tested on the same gastric fistula t. The dose of all extracts was 3 mg/kg. If this was

TABLE I. Gastrin activity expressed as histamine units (HU) per g tissue, in different layers of the antral mucosa and submucosa of the cat. Sections were cut parallel to the mucosal sur surface and pooled from 5, 9 and 11 antra in experiments 1 2 and 3 respecti ely

Experiment	Thickness of tissue (from mucosal surface) μ	Weight of tissue g	mg extract / g tissue	HU / mg extract	HU / g tissue
1	240	1.59	38 0	<1 0	< 38
	240	1 49	54 6	3.3	180
	240	1.62	56.1	3.8	213
	240	1 62	36.4	<1.0	< 36
2	240	3.17	12.5	<1 7	< 21
	160	2.03	30.4	2.5	76
	160	1.99	48.1	4.7	226
	160	1.95	40.5	2.5	101
	240	2.87	9 7	<1.0	< 10
3	240	3.95	55.6	<1.0	< 56
	120	1.89	64.2	3.2	205
	120	1.93	66.6	3.3	220
	120	1.90	70.3	3.7	260
	280	3 05	45.5	<0.7	< 31

insufficient to produce an acid response equal to or greater than the response to the small reference dose of histamine which has to exceed 0.10 meq/hr the response of the extract was recorded as less than that to histamine. The gastrin activity was expressed in histamine units (HU) per mg extract and in HU per g of mucosa. The secretory activity of an extract is 1 HU if 1 mg of the extract per kg b.w elicits the same 1-hr acid response as 0.001 mg of hista mine dihydrochloride per kg b.w both substances infused i.v for 15 min (Uvnäs and Emås 1961)

The ability of some extracts of the aboral corpus mucosa to potentiate the acid sham feeding response in an antrectomized Pavlov pouch dog (Olbe 1964) was also tested. The extract was infused i. during 30 min, beginning 15 mi fter the start of the sham feeding. The acid output was determined by titrating the pouch secretion with 0 01 N NaOH, using phenol phthalein as indicator The 1.5 hr acid sham feeding response with and without infusion of standard gastrin extract in subthreshold dosage or with infusion of the extracts of distal corpus mucosa was determined (T ble IV) I a few experiments spontaneous acid secretion appeared before the sham feeding and such experiments hav not been included

Results

Localization of gastrin activity in the ant al muco When the antral mucosa was dissected from the muscular coat w th blund-edged scissors, the splitting took place within the submucosa. In 23 histological sections ut perpendicular to the surface of the antral mucosa from 5 cats, the height f th gland region amounted to 76 per cent (range 63—84) of the total thickn of the pr parations The height of th gland region varied even in the same section I 20— such sections from each dog antral mucosa, the height of the gland regi consti tuted n dog A 44 per cent (range

TABLE II. Gastrin activity expressed as histamine units (HU) per g tissue, in different layers of antral mucosa and submucosa of the dog.

Dog	Thickness of tissue (from mucosal surface) μ	Weight of tissue g	mg extract / g tissue	HU / mg extract	HU / g tissue
A	280	1 02	70.6	1 0	71
	280	0.84	94.8	2.8	263
	280	1 11	77 4	3 0	232
	280	1 11	92.5	0 7	62
		5.93	44 7	1.8	81
B	320	0.98	18.3	<0 7	<13
	320	0.91	63.6	0 7	44
	320	0.99	65 6	0.8	53
	320	1 04	47 1	1.3	61
		2.23	34 1	<0 7	< 23
C	320	1.98	19 7	0 7	14
	320	1 48	62.7	1.5	94
	320	1 57	67 0	0 7	47
	320	1.58	53.7	<0 7	< 38
	320	1 48	31.9	0.7	22
		0.58	24 0	<0.7	< 17
	315	1 84	43.4	0.8	35
	315	1 42	74.8	1.5	112
	315	1 61	65.3	1..	78
	315	1.60	59.9	<0 7	< 42
	315	1 43	52.4	0 7	37
	315	1 17	33 1	0 8	27
		0.82	27 0	1 0	27

33—63) in dog B 48 per cent (range 40—57) in dog C 55 per cent (range 39—75) and in dog D 49 per cent (range 31—65) of the total thickness of the preparations. The total thickness of the antral mucosa (measured in μ) was not determined in the histological sections, since these sections had been shrunk by the fixation, but was calculated from the cutting procedure in the cryostat and by weighing the uncut part of the tissue cylinders (Table I and II) Knowing the total thickness of the antral mucosa cylinders in μ and the height of the gland region as a percentage of the total thickness, the height of the gland region in μ could be calculated (Fig 1 and 2) The findings in the control histological sections, taken at various depths in the tissue cylinders of the dog antra—i on the borderlines between the different pools of sections—were in good agreement with those expected from the calculated height of

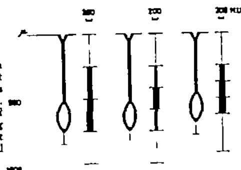


Fig 1 Gastrin activity indicated by the width of the vertical bars, in sections of cat antra cut parallel to the mucosal surface. The activity is expressed as histamine units (HU) per g tissue. In three experiments the sections from 5 9 and 11 antra, respectively were pooled. Knowing the mucosal thickness, the mean gland height and its range were calculated from histological sections cut perpendicular to the mucosal surface.

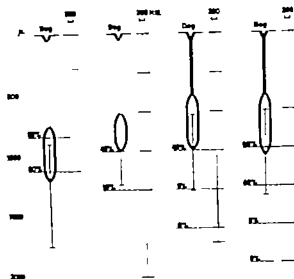


Fig. 2 Gastrin activity indicated by the width of the vertical bars, in sections of 4 dog antra cut parallel to the mucosal surface. The activity is expressed as histamine units (HU) per g tissue. Knowing the mucosal thickness, the mean gland height and its range were calculated from histological sections cut perpendicular to the mucosal surface. At various depths of the mucosa, sections cut parallel to the mucosal surface were examined histologically and the percentage of the area occupied by gland tissue is shown in the figure

the gland region. Thus the control sections taken around the calculated mean bottom of the gland region had gland tissue occupying about 50 per cent of the area, with the exception of dog D (Fig. 2)

In all 3 series of mucosae from cat antra the gastrin activity was confined to the basal 2/3 of the gland tissue. No gastrin activity was demonstrable in the superficial 1/3 of the gland tissue or in the submucosal tissue (Fig 1, Table I). It is to be noted that although this submucosal tissue should contain some gland tissue from the bottom of long gland crypts (Fig 1) no gastrin activity was found there. 3 extracts from the superficial part of the mucosa and 3 extracts from the submucosal tissue were

mixed with a standard extract of known gastrin activity The mixture of extracts was injected in a conscious gastric fistula cat and gave the same acid response as did the standard extract alone.

In the dog antral mucosa the maximal gastrin activity was found in the basal /3 of the gland tissue region, as in the cat antral mucosa. Again the maximal gastrin activity was obtained above the deepest part of the gland region, with the exception of dog B (Fig. 2) However small amounts of gastrin activity were found both in the superficial part of the mucosa—in 3 out of 4 dogs—and in the submucosa—in 1 out of 3 dogs—(Fig 2, Table II)

The part of the antral submucosa that was attached to the muscular coat, was excised in 3 cats and 3 dogs. Extracts from this submucosal tissue did not show any gastrin activity

Gastrin activity in the aboral corpus mucosa. The aboral 2 cm of corpus mucosa from 3 dogs and 14 duodenal ulcer patients was extracted for gastrin.

The extracts from the dog corpus mucosa were tested on a conscious gastric fistula cat. No gastrin activity was found (Table III)

Extracts from the corpus mucosa from 10 ulcer patients were tested on a gastric fistula cat In 2 of these extracts gastrin activity was found (Table III) Mucosal extracts from the antral side of the antrum-corpus boundary in 2 patients showed definite gastrin activity (Table III)

Extracts of the aboral 2 cm of corpus mucosa from 11 ulcer patients were i.v in

TABLE III. Gastrin activity expressed as histamine units (HU) per g tissue in the aboral 2 cm of corpus mucosa of dogs and duodenal ulcer patients. In 2 patients (VF and KB) the gastrin activity in the antrum mucosa was also determined.

Dog no. or patient initials	mg extract / g tissue	HU / mg extract	HU / g tissue
Dog 1	55.8	<1 0	< 56
Dog 2	55 1	<1 0	< 55
Dog 3	48.4	<0.7	< 34
ST	46.5	0.7	< 33
AL	49 2	0.7	< 34
AA	64 3	<0 7	< 45
EG	69 1	<0.7	< 48
EP	50 9	<0 7	< 36
GP	50 1	1 4	70
OF	53 7	<0 3	< 16
TJ	78 1	<0.3	< 23
VF	42 7	<0.7	< 30
VF (antrum)	36 1	1 7	61
KB	52 7	1.8	95
KB (antrum)	46 4	3.7	165

TABLE IV 1.5 hour acid sham feeding response in antrectomized Pavlov pouch dog with and without concomitant 30 min i. infusion of extracts from the aboral corpus mucosa of duodenal ulcer patients or of standard gastrin extract in subthreshold dosage.

Infused extract in mg and patient initials	Acid response in meq Exp. no. 1	2	3	4	5
0	0.02	0.13	0.01	0.02	0
140 mg ST	0.04	0.82			
160 mg AL	0.03				
190 mg AA	0.06	0.01			
145 mg EG	0.03				
177 mg EL	0.85				
120 mg GP	0.28	0.95			
190 mg GF	0.15				
135 mg RN	0.05				
153 mg VJ	0.57				
95 mg AP	0.01				
127 mg TJ	0.06				
120 histamin units of a standard gastrin extract	0.70	0.25	0.96		

Acid response appeared following the end of the infusion.

fused concomitantly to sham feeding in a Pavlov pouch dog from which the gastrin releasing regions had been removed. Sham feeding alone produced only a very small acid response (Table IV) I v infusion for 30 min of an antral extract of known *gastrin activity in subthreshold* dosage augmented the acid sham feeding response (Table IV) Three of the eleven extracts from human distal corpus mucosa increased the acid sham feeding response (extracts EL, GP and VJ) and the action of one extract (ST) was uncertain (Table IV)

Seven of the extracts from human aboral corpus mucosa were tested on the conscious gastric fistula cat as well as on the sham fed antrectomized Pavlov pouch dog. The results were in good agreement (Table III and IV).

Discussion

In the present study the maximal gastrin activity within the antrum of cat and dog was found in the glandular tissue region and not in the submucosa. Our finding does not support the hypothesis that the gastrin releasing cell might be a modified ganglion cell of the Meissner's plexus in the submucosa. This hypothesis was proposed on the basis that surgical (Baugh *et al.* 1958) or chemical (Woodward *et al.* 1963) destruction of submucosal elements of the antrum inhibited the gastrin release from the antrum. *However such findings do not necessarily imply that the* gastrin re

leasing cells have been destroyed, but can also be explained as being due to destruction of nervous reflex pathways involved in the release of gastrin, passing through the submucosa. The latter idea is supported by the observation that injection of carbolic acid into the antral submucosa inhibited the gastrin release induced by a liver solution in the antrum, but not that induced by acetylcholine in the antrum (De la Rosa *et al.* 1957). Present evidence favours the view that the release of gastrin from the antrum by all known local releasers, with the exception of cholinergic drugs, involves a nervous reflex mechanism (see review by Olbe and Elwin 1966).

The maximal gastrin activity was found in the basal 1/3 of the gland region of the antral mucosa. The deepest part of the gland region, however, contained no gastrin activity in the cat and only low activity in the dog (Fig. 1 and 2). The superficial 1/3 of the antral mucosa of the cat contained no gastrin activity but again in the dog a low activity was found in this region in 3 dogs out of 4. The submucosa of the cat antrum contained no gastrin activity but the submucosa of at least one dog antrum (dog D) contained small amounts of gastrin activity. The low gastrin activity observed in the superficial part of the mucosa and in the submucosa might be due to a masking of gastrin activity by the presence of an inhibitor of acid secretion in those regions. We found, however, no evidence for the presence of any such inhibitor in the superficial part of the antral mucosa or in the submucosa, since a mixture of an extract from these regions and a standard gastrin extract produced the same acid response as did the standard gastrin extract alone. The gastrin cells therefore seem to be most numerous in the middle and basal part of the gland crypt region of the antrum, possibly with a wider scattering in the dog than in the cat. This location of gastrin in the antral mucosa corresponds roughly to the location of secretin in the duodenal mucosa, where maximal secretin activity was found in extracts from the transitional zone between the villous layer and the crypts of Lieberkühn (Kraaij *et al.* 1964).

It was recently reported (Solcia, Vassallo and Sampietro 1967) that an endocrine-like cell, displaying all the morphological features of protein secreting cells, has been found in the antral mucosa of some species. Within the antral mucosa of guinea-pig and rabbit the cells were scattered mainly in the epithelium of the body of the gland crypts, but in man and horse they were located more superficially. There were no reports of the localization of the cell in cat and dog. However, the localization of the endocrine-like cell in the antrum of other species and the distribution of gastrin activity in the present study seems to be correlated, supporting the idea that this cell might be the gastrin cell.

The antrum-corpus boundary can be visualized during operation by a pH indicator technique, and this boundary between acid secreting and non acid secreting mucosa coincides with the histological antrum-corpus boundary in dog and man (Olbe 1963, Berstedius and Emås 1964). In the present study the 2 cm of corpus mucosa situated immediately oral to the antrum-corpus boundary was excised from dogs and 14 duodenal ulcer patients. This corpus mucosa was prepared by the Korman method to obtain gastrin extracts. The acid secretory activity of the extracts was

tested on conscious gastric fistula cat and or on a sham fed antrectomized Pavlov pouch dog. The extracts from the dog corpus mucosa showed no gastrin activity but 4 of the 14 extracts from corpus mucosa of the duodenal ulcer patents contained gastrin activity Seven of the human extracts were tested both for acid production in a conscious gastric fistula cat and for the ability to potentiate the acid sham feeding response in an antrectomized Pavlov pouch dog Both test methods gave the same result, indicating an equivalence between these two methods for biological detection of gastrin activity Since the majority of the corpus extracts did not contain gastrin activity it seems reasonable to conclude that the gastrin cells are only distributed on the antral side of the antrum-corpus boundary but that this boundary is sometimes rather irregular In fact the human antrum-corpus boundary has been histologically demonstrated as irregular often dentate, and sometimes deeply notched (Landboe-Christensen 1944) which might explain why some extracts from the aboral corpus mucosa of man in the present study contained gastrin activity Thus the gastrin cells may be distributed on both sides of the antrum-duodenum boundary since gastrin-like activity can be extracted from at least the proximal part of the duodenum (Emås and Fyrö 1968) but only on one side of the antrum-corpus boundary From a practical point of view any resection of the human antrum should include at least the aboral 2 cm of corpus mucosa with the sometimes notchy antrum-corpus boundary and probably the duodenal bulb, in order to minimize the gastrin releasing regions.

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References

Bydom, O. M., J. L. Bravo J. Barcena and L. R. Dragstedt Studies on the site and mechanism of gastrin release. A h Surg 1958 76 441—446.

Bergström H. and A. Badoni, Peroperative determination of the boundary between the gastric antrum and fundus. Act ? scand. 1964 128 526—529

De la Rosa, C E. R. Woodward and L R Dragstedt Localization of the gastrin producing cell. Surgical Forum 1965 16 327—329

Emås, S. and B. Fyrö, Gastrin-like activity in different parts of the gastrointestinal tract of the cat. Act physiol. scand. 1968. 74 359—367

Komarov S A Gastrin. P oc Soc exp Biol. (N Y) 1938. 38 514—516

Krawitt E. L., G. R. Zimmerman and J. A. Clifton, Location of secretin in dog duodenal mucosa. Amer J Physiol. 1966 211 935—938.

Landboe-Christensen, E., Extent of pylorus zone in the human stomach Act path microbiol. scand 1944 S ppl 54 671—692.

Olbe, L., Significance of vagal release of gastrin during the nervous phase of gastric secretion in dogs. Gastroenterology 1963 44 463—468.

Olbe, L., Potentiation of sham feeding response in Pavlov pouch dogs by subthreshold amounts of gastrin with and without acidification of denervated antrum. Act physiol. scand 1964. 61 244—254

Olbe, L. and C E Elwin Mechanisms stimulating antral release of gastrin Postgrad at Gastroenterology Conf. in Glasgow 1965. Baillière Tindall & Cassel, London 1966. 140—151

Solcia, E., C Vassallo and R. S ? Endocrine cells in the antro-pyloric mucosa of the stomach Z. Z Forsch. 1967 81 474—486.

Uvnäs, B. and S. Emås, A method for biologic assay of gastrin Gastroenterology 1961 40 644—648.

Woodward E. R., C. L. Park, H. Schapiro and L R Dragstedt Significance of Meissner's plexus in the gastrin mechanism. A ? S g 1963 87 51 —515

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Projections of the Eye and the Neck Region on the Anterior Suprasylvian Cerebral Cortex of the Cat

By

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Abstract

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The overlap between projections of visual, auditory, cutaneous and muscle afferents to the cerebral cortex of the anterior suprasylvian sulcus was studied in cats anesthetized with chloralose. The visual projections were found near the junction between the anterior and the middle suprasylvian sulcus. They overlapped in rostral direction with the auditory projection area in the lower bank of the anterior sulcus. The overlap was, however, not complete and large click potentials, but no responses to light flash, were recorded in the lower bank just caudal to the locus of the Group I muscle afferents. Low threshold cutaneous and high threshold muscle afferents from the neck projected to the anterior suprasylvian and ectosylvian gyri and to the upper bank of the anterior suprasylvian sulcus. Low threshold muscle and cutaneous neck afferents projected to the postcruciate dimple. A hypothesis concerning the function of this cortical region is discussed.

The present study concerns the cerebral cortex of the anterior suprasylvian sulcus and bordering parts of the anterior suprasylvian and ectosylvian gyri in the cat. This part of the cortex is located between the primary somatosensory projection areas (Adrian 1940, 1941, Marshall, Woolsey and Bard 1941) and the primary auditory projection areas (AI and AII, cf. Woolsey 1960). It is characterized by its reception of projections from several different sensory organs. This fact was first pointed out by Mickle and Ades (1952) who studied a part of the above mentioned region and suggested the name *composite sensory area* for that part receiving somatosensory, auditory and vestibular projections. The afferent inflow was further studied by Landgren, Silfvenius and Wolsk (1967 a, b) who showed that the region received projections from muscle afferents belonging to Group I, II and III, from low threshold joint afferents, from skin afferents, from cochlear and from vestibular afferents. The location and overlap of the projection fields were mapped in some detail. It was now considered important to investigate whether the region was a true composite sensory area receiving input from all sensory spheres such as was

the case with the association areas described by Albe Fessard and Rougeul (1958) Buser and Borenstein (1959) and by Thompson, Johnson and Hoopes (1963) The overlap of the previously studied projections with those from the eye and the neck region was investigated and the result will be presented in the following report.

Methods

The methods were described by Landgren, Silfvenius and Wolsk (1967) Ten cats anesthetized with chloralose (70 mg/kg i.v.) were used for the experiments. The following contralateral nerves were dissected and prepared for graded electrical stimulation The deep radial nerv the superficial radial nerve the sural nerves, skin nerves from 1st to 5th cervical roots, the nerves of m. splenius

The auditory stimulus was free field click delivered binaurally from loudspeake mounted about 10 cm from the contralateral pinna. The click was produced by feeding 100 μsec square pulse from one of the stimulators into the loudspeaker

The visual stimulus was single flash deli red to the contralateral atropinized eye from a gas discharge tube activated by condensor discharge. The flash was conveyed from the lamp to the eye via plexiglass rod. The lamp was placed in sound insulating box about 1.5 m from the cat head It was noiseless to the experimenters, and routine checks were made by blocking the flash with an opaque screen.

The maps of the projection fields show the extent of the cortical area within which the evoked potentials were initially positiv when recorded from the cortical surface with silver ball electrode, and initially negativ when recorded from the underlying deep cortical layers with penetrating microelectrode. Areas with initially positive evoked potentials recorded from the surface as well as from layers III to V of the cortex, i.e. the click responses in the anterior suprasylvian gyrus, were not included (cf Thompson, Johnson and Hoopes 1963) The border of the projection area indicates position where the amplitude of the evoked potential was less than 20 % of that of the maximum response.

Results

A. *Visual projections t the anterior suprasyl ian r gion*

Visual projections to the middle suprasylvian gyrus were previously described by Buser Borenstein and Bruner (1959) These projections belong to the association areas, which receive afferent paths ndependent of the primary projection systems. This independence was emphasized by the results of Thompson, Smith and Bliss (1963) who also demonstrated the existence of a primary visual projection area in the medial bank of the middle suprasylvian sulcus. Because of these previous results it was expected to find responses evoked by visual stim li near the junction between the middle and the anterior part of the suprasyl ian gyrus Th rostral extent of the visual projections and their overlap with the uditory estibular and somatosensory fields in the nterior suprasylvian sulcus was, however not sufficiently studied. This is not only a question of anatomical detail but has f nctional implications, because of the information it provides concerning the afferent convergence within minute cortical areas.

This visual projection area is illustrated by ertical hatching n the cortical diagram of Fig. 1 It ends just rostral to the upper knee-shaped bend of the supra syl ian sulcus at the junction between ts middle and anterior limb The field ex tends slightly furthe rostrally in the lower than in the upper bank of the sulcus It thus overlaps with the uditory projection area in the lower bank (horizontal

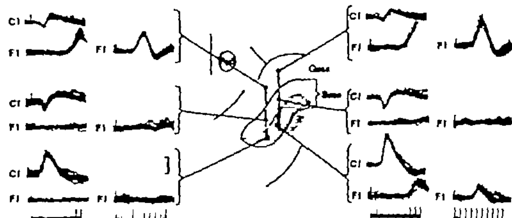


Fig. 1. *Records of focal potentials evoked by loudspeaker click (Cl) and a light flash (Fl) in cortical layers III to V.*
Recording sites are indicated on the diagram. Negativity is recorded upwards. Time scale 5 msec for the left and 5+10 msec for the right columns. Voltage scale 0.5 mV.
Ped = postcrucial dimple. Gsaa = gyrus suprasylvius anterior. Ssaa = sulcus suprasylvius anterior opened up to display the upper bank (a) and the lower bank (b). Stippling = projection fields of the Group I muscle afferents from the contralateral forelimb. Vertical hatching = visual projection field. Horizontal hatching = auditory projection field.

tching) but the two fields are not coextensive. The click thus evoked large focal potentials between the visual field and the projection area of the Group I muscle afferents (stippling).

Fig. 1 also shows responses to visual and auditory stimuli evoked in cortical layers III to V at selected recording sites in a typical experiment. The auditory responses were of the type described by Landgren, Silfvenius and Wolsk (1967 b). In agreement with the observations of Thompson, Johnson and Hoopes (1963) they were found to be initially positive in the deeper layers of the suprasylvian gyrus as well as in the upper bank of the sulcus. In layers III to V of the lower bank, however, the click evoked a large negative focal potential.

Such a potential was recorded in both lower bank positions illustrated in Fig. 1. The posterior one also showed a small initially negative potential evoked by the flash; no visual response was found in the anterior recording site.

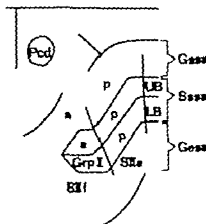
Confirming the observations of Mickle and Ades (1953) the latency of the click response in the lower bank was shorter than that in the primary auditory projection area (AI) of the same animal. It was generally about 7 msec and the shortest observation was 6.? msec. Judging from the point of view of the latency the auditory projections of the anterior suprasylvian sulcus are primary in nature. This is, however, not the case with the visual projections. As shown in Table I all responses evoked by the light flash were of long latency, generally about ?0 msec and thus approximately 5 to 10 msec longer than that seen in the primary visual projection area.

TABLE 1 Latencies and thresholds of potentials evoked in the cerebral cortex by light flash, loud speaker click and electrical stimulation of contralateral nerves from the neck

Cortical area		Flash latency	Click latency	Neck skin		Neck muscle	
				latency	threshold	latency	threshold
Pcd (caudo-medial)		—	—	4.5—5.5	0.8—1.3	3.5—7.5	1.1—1.9
Gssa		—	—	4.0—7.0	0.8—1.5	5.5—9.0	4.0—10.0
Gssa	p	21.0—28.0	—	3.5—8.0	0.7—1.4	—	—
UB		—	—	4.2—6.0	0.9—1.3	5.0—8.0	2.0—6.0
UB	p	21.0—23.0	—	4.8—6.5	1.0—1.5	9.0	7.8
LB	GrpI	—	—	—	—	—	—
LB	p	20.0—33.0	6.2—9.0	5.0—9.0	0.9—1.6	9.0	7.8
	Gesa			4.0	1.0		
	A I		8.0—10.0				
	V I	11.0—14.7					

Latencies in msec. Thresholds in multiples of stimulus strength evoking threshold response in the afferent nerve (T). The figures give the range of observations.

The diagram gives the location of the cortical areas referred to in the table. Pcd = post cruciate dimple. Gssa = Gyrus suprasylvius anterior, a = anterior, p = posterior subdivision. Sssa = Sulcus suprasylvius anterior. UB = Upper bank, LB = lower bank, Grp I = locus of projections from contralateral Group I muscle afferents. Gesa = Gyrus ectosylvius anterior. SIIf = forelimb projection to second primary somatosensory area. SIIa = SII of *Carreras* and *Andersson* (1963). A I = primary auditory cortex. V I = primary visual cortex.



B. *Cutaneous and muscle afferent projections from the neck to the anterior suprasylvian gyrus*

The projections of afferents from the neck to a cortical region receiving input from the vestibular apparatus (*cf.* Landgren, Silfvenius and Wolsk, 1967b) was considered interesting because the peripheral receptors of both systems are excited by movements of the head, and evidence of convergence may be functionally significant.

Cutaneous afferents were selected from branches entering through dorsal roots C1 to C5. These afferents include fibres from tactile receptors because responses to gentle brushing of the fur were recorded from the cut peripheral end of the branches.

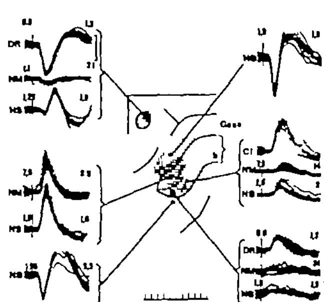


Fig. 2. Records of potentials evoked by electrical stimulation of the deep radial nerve (DR) nerves to neck muscles (NM) and neck skin (NS). Recording sites are indicated in the diagram. The records from the sulcus (a and b) show focal potentials from cortical layers III to V, the other records were obtained from the cortical surface. The figures to the left give thresholds of the cortical potentials in multiples of the threshold of the afferent volley; the figures to the right give stimulus strength used to evoke the recorded potential. Time scale 5 msec. Voltage scale 0.2 mV for left column and for upper and lower groups of right column, 0.4 mV for middle group of right column. Vertical hatching = NS projection field. Horizontal hatching = NM projection field.

The receptive fields were mapped and found within the dermatomes of C1 to C5 as described by Hekmatpanah (1961).

The projection areas of the low threshold cutaneous afferents from the neck are indicated by vertical hatching in the diagram of Fig. 2. Evoked potentials of large amplitude were found in the anterior suprasylvian gyrus, in the upper bank of the sulcus and in the anterior ectosylvian gyrus (*cf.* Fig. 2). As seen in Table I the latencies of these potentials were short with minimum observations around 4 msec in the anterior subdivisions. In the lower bank of the sulcus the evoked potentials were smaller in amplitude and had longer latencies. No response was recorded within the locus of the Group I afferents from the contralateral forelimb. The cutaneous neck projections therefore form two foci separated by the lower bank of the anterior suprasylvian sulcus. A third focus was found near the postcruciate dimple. This area was only a couple of mm in diameter and overlapped with the Group I muscle afferent projections to this region. The potentials evoked there by the neck afferents were of short latency but the amplitude was small compared to that of the suprasylvian region (*cf.* Table I and Fig. 2).

A considerable overlap was found between the projection fields of cutaneous branches entering through the rostral and the caudal cervical roots. In agreement with the findings of Celesia (1963) the fields of the rostral roots extended further rostrally on the anterior suprasylvian gyrus than those of the caudal roots.

Responses evoked by muscle afferents from the contralateral splenius muscle were observed, but they were small and it was often necessary to use a short train of shocks to improve the amplitude of these potentials. Their cortical fields are indicated with horizontal hatching in the diagram of Fig. 2. These projection areas

were always found within those of the cutaneous afferents. All responses observed in the suprasylvian gyrus and sulcus were evoked by high threshold splenius afferents. In most of the experiments the amplitude maximum of the evoked potential was found in the upper bank of the sulcus. In two experiments potentials evoked by Group I splenius afferents were recorded from the area located in the postcruciate dimple. Thresholds and latencies of the potentials are given in Table I.

The observation of a separate projection area for cutaneous and low threshold muscle afferents near the postcruciate dimple disagrees with the findings of Celesia (1963) who did not describe any projection from the rostral cervical segments to this region. The difference may be due to different anaesthesia used in the two series of experiments. The present observation of a focus in the dimple is, however, expected when considering the results of Oscarsson, Rosén and Sulg (1966) who demonstrated convergence between projections of Group I muscle afferents and cutaneous afferents from the contralateral forelimb to th postcruciate dimple.

Discussion

The present series of experiments adds further details concerning the minute organization of the anterior suprasylvian fold. One of these details helps in understanding the function of the auditory projections to this region. The anterior part of the auditory field does not receive visual projections. The information bout the periphery utilized in this area is thus supplied by the auditory estibular and somatosensory systems. The hypothesis that the area could play a role in the animal's orientation against sound stimuli was suggested by Landgren, Silf emus and Wolsk (1967 b) and behavioural tests of the validity of this hypothesis are in progress. The visual projections overlapping the auditory field near the upper bank of the anterior suprasylvian sulcus may be invol ed in mechanisms controlling the orientation of the eyes towards suddenly occurring sounds. If this hypothesis is alid t would be expected to f nd afferent projections from external eye muscle afferents to the suprasylvian fold. One would further expect to find an efferent path from this cortical area to the eye muscles. Preliminary experiments (Landgren and Silf enius, unpublished) show that activity in the external eye muscle could be evoked by electrical stimulation in the region of the anterior suprasylvian sulcus. Additional experiments are however required to localize the origin of these motor effects.

The observation of isual projections adjacent to and possibly overlapping w th the projections from the vestibular nerve is not unexpected, if one considers the co-excitation of the two systems during head movements. The importance of mechanisms of correlation for isual and estibular information subserving the control of movements was previously emphasized by Jung (196_).

It may be i teresting to compare the features of the anterior suprasylvian fold wit those of the nearby anterior middl suprasylvian association area (AMSA of Thompson *et al.* 1963). Both regions recei a pol sensory input, but—with the

exception of the visual responses—the potentials evoked in the suprasylvian fold differ considerably in latency from those recorded in AMSA. Several loci in the fold, i.e. the Group I locus, the auditory area and the vestibular area, are characterized by short latency responses. The latencies are in fact as short as or even shorter than latencies observed in any other cortical projection area. From this point of view the projections to the suprasylvian fold may be considered to be "primary". A certain somatotopical organization was found in the fold by Landgren, Silfvenius and Wolsk (1967 a) in the sense that forelimb and hindlimb responses dominate in different loci. The somatotopy of this region is, however, not as detailed as that found in the primary projection areas. Further knowledge concerning somatotopical relations and modality specificity may, however, be obtained in single unit studies.

The question whether certain loci in the fold receive corticocortical connections from the primary projection areas cannot be answered at present. Imbert, Bignall and Buser (1966) have demonstrated such connections between the primary visual cortex and the association areas. It was pointed out by Landgren, Silfvenius and Wolsk (1967 a) that the latencies of the potentials evoked in the fold by the cutaneous afferents from the forelimb were about two msec longer than those of the responses in the primary somatosensory areas. This observation was confirmed and was found to be true also for the cutaneous projections from the neck, when latencies of potentials recorded in the lower bank were compared with those found in the [illegible] of these projections to the anterior suprasylvian gyrus. There is obviously room for additional synapses in the cutaneous paths to the fold but their nature is still unknown.

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References

Adrian, E. D. Double representation of the feet in the sensory cortex of the cat. J. Physiol. Lond. [illegible] 16P.

Adrian, E. D. Afferent discharges to the cerebral cortex from peripheral sense organs. J. Physiol. Lond. [illegible]

Albe-Fessard, D. and A. R[illegible]. Activités d'origine somesthésique évoquées sur le cortex non-spécifique du chat anesthésié au chloralose: rôle du centre médian du thalamus. Electroenceph. clin. Neurophysiol. [illegible]

Buser, P. and P. Borenstein. Réponses somesthésiques, visuelles et auditives, recueillies au niveau du cortex "associatif" suprasylvien chez le chat curarisé non anesthésié. Electroenceph. clin. Neurophysiol. [illegible]

Buser, P., P. Borenstein and J. Bruner. Etude des systèmes "associatifs" visuels et auditifs chez le chat anesthésié au chloralose. Electroenceph. clin. Neurophysiol. [illegible]

[illegible] and S. A. [illegible]. Functional properties of neurones of the anterior ectosylvian gyrus of the cat. J. Neurophysiol. [illegible]

[illegible], G. G. Somatotopic organization of cortical afferent areas in the cat. J. Neurophysiol. [illegible]

[illegible], J. Organization [illegible] J. Neurophysiol. [illegible]

Imbert, M., G. E. Bignall and P. Buser. Neocortical interconnections in the cat. J. Neurophysiol. [illegible]

J[illegible], R. Optic and [v]estibular factors in motor coordination. *P[r]oc. [I]nt. [illegible] physiol. Sc.* Leiden 1962. pp. 518—525

Landgren, S., H. Silfvenius and D. Wolsk, Somatosensory paths to the second cortical projection [a]rea of the Group I muscle afferents. *J. Physiol.* (Lond.) 1967a. *191* 543—559

Landgren, S., H. Silfvenius and D. Wolsk, Vestibular cochlear and trigeminal projections to the cortex in the anterior suprasylvian sulcus of the cat. *J. Physiol.* (Lond.) 1967b. *191* 561—573

Mickle, W. A. and H. W. Ades, A composite sensory projection area in the cerebral cortex of the cat. *Amer. J. Physiol.* 1952. *170* 682—689

Marshall, W. H., C. N. Woolsey and P. Bard, Observ[a]tions on [c]ortical somatic sensory mechanisms of cat [a]nd monkey. *J. Neurophysiol.* 1941 *4* 1—24

Mickle, W. A. and H. W. Ades, Spread of evoked cortical potentials. *J. Neurophysiol.* 1953 *16* 608—633

Oscarsson, O., I. Rosén and I. Sulg, Organization of neurones in the cat cerebral cortex that are influenced from Group I muscle afferents. *J. Physiol.* (Lond.) 1966. *183* 189—210

Thompson, R. F., R. H. Johnson and J. J. Hoopes, Organization of auditory, somatic sensory and [v]isual projection to association fields of cerebral cortex in the cat. *J. Neurophysiol.* 1963 *26* 343—364

Thompson, R. F., H. E. Smith and D. Bliss, Auditory, somatic sensory and [v]isual response interactions and interrelations in association and primary cortical fields of the cat. *J. Neurophysiol.* 1963 *26* 365—378.

Woolsey, C. N., Organization of cortical [a]uditory system. A Review [a]nd Synthesis. *Neural Mechanisms of the Auditory and V[e]stibular Systems.* Ed. Rasmussen, G. L. and W. F. Windle. C. C. Thomas, Springfield, Ill. 1960 pp. 165—180

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Activity of Pilomotor Muscles of Single Tactile Hairs in the Cat

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Abstract

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Contractions of the pilomotor muscles of individual carpal tactile sinus hairs in the cat were studied by simultaneous recording of the movement of a single hair by means of a capacitance meter and of the electrical activity during the muscle contraction, as recorded by an external metal microelectrode. Single shock stimulation of the nerve gave rise to a twitch contraction, whereas repetitive stimulation caused summation of contractions at frequencies higher than 0.2/sec and fusion at frequencies of 1/sec or more. The electrical response consisted of a slow potential and a superimposed spike. These components were both shown to be dependent on the stimulus strength and to be facilitated on repetitive stimulation. At stimulus frequencies higher than 3/sec the spike gradually declined in amplitude and was substituted by a rhythmic oscillation at the same rate as the stimulus. Even when no spike was recorded the contraction was sustained. Comparisons are made between the electrical activity elicited in the pilomotor muscles and the junction potentials and spikes recorded in other types of smooth muscle.

The tactile sinus hairs on the cat's forelimbs are surrounded by a strong smooth musculature which corresponds to the pilomotor muscles of the ordinary hairs (Fritz 1909, Nilsson and Skoglund 1965). This musculature consists of muscle bundles, 50—100 μ in diameter, which are oriented both horizontally around the lower part of the sinus and vertically reaching up toward the epidermis. They are richly innervated by adrenergic fibers (Fuxe and Nilsson 1965). In the course of further studies of the mechanoreceptors of these carpal tactile hairs, contraction of the muscles induced by nerve stimulation was found to influence the receptor response (Nilsson, unpublished observations), and this finding prompted a closer study of the behavior of these pilomotor muscles.

It has earlier been established that the pilomotor muscles of the ordinary hairs are adrenergically innervated and that they respond with a twitch contraction to single shock stimulation of sympathetic nerves, whereas repetitive stimulation at low frequencies results in mechanical summation and a fused response (Rosenblueth and del Pozo 1942, Hellman 1963). The electrical activity during contraction of the pilomotor muscles has been studied on cat tail preparations by Rosenblueth and collaborators (*cf.* Rosenblueth and del Pozo 1942) but the potentials obtained in

their experiments were complex and difficult to analyze since they recorded the sum of the activity from the pilomotor muscles present in a large skin area.

Pilomotor muscle contractions have also been studied in connection with studies of the transmission in the sympathetic nervous system (cf. e.g. Burn et al. 1959, Wolner 1965) but these publications did not provide any further data on the physiological properties of the muscles.

The pilomotor musculature of the ordinary hairs cannot easily be studied since it is a minute smooth muscle structure with no attachments to rigid structures. The corresponding muscles of the carpal tactile hairs are far stronger and thus more readily available for physiological experiments. This paper will give an account of such experiments designed to study the contraction properties of these muscles in the cat as reflected in movements of a single hair and to illustrate the electrical activity during contraction, as recorded from a small part of the musculature by means of an external metal microelectrode.

Methods

The cats were anesthetized with Nembutal (40 mg/kg bw). The ulnar nerve branch to the skin area containing the carpal tactile hairs was exposed in the lower leg. After stitching the edges of the skin to a skin retractor and covering the area with liquid paraffin, the nerve was cut and stimulating electrodes placed on its distal part, about 2 cm proximal to the pilomotor musculature. The cat was kept warm by means of a heating lamp and the temperature in the paraffin pool was 33—35 °C (cf. Hellman 1963 b).

Because of the complicated arrangement of the musculature around the root of the sinus hairs, contraction has a twofold effect on the hairs: they stand up and move sidewards in different directions. This complex movement was recorded by measuring the change in capacitance taking place when a hair moved away from a probe consisting of a metal wire placed close to and parallel with the outermost 2—3 mm of the hair which was cut to a length of about 10 mm. The small capacitance variations were recorded by a high-sensitivity capacitance meter. In order to increase the sensitivity to incremental capacitance changes a very thin copper wire (diameter 0.05 mm) connected to earth was attached along the hair. The effect of this wire on the hair movements, and hence on the experimental results, was negligible. On maximal contraction the outermost tip of the hair moved maximally 5 mm away from the probe. The recording of the movements was linear only over part of this distance.

The electrical activity during contraction was recorded by means of a glass-insulated platinum-iridium electrode (Wolbarsht and Wagner 1963) of a tip diameter of 1—3 μ and an impedance of 40—90 kΩ at 1000 c/s, which was inserted through an incision in the skin close to the sinus hair. A reference Ag-AgCl electrode was placed in contact with the subcutaneous layer of the skin, the interelectrode spacing being 1 cm. The microelectrode did not noticeably influence the movement of the hair. The Grass model P6 preamplifier was equipped with a symmetrical low-pass L-C filter (attenuation 50 dB at 1085 kc/s, no attenuation below 50 kc/s) at the input in order to prevent blocking of the amplifier by the high-frequency carrier from the capacitance meter.

Results

A typical example of the movements of a sinus hair on supramaximal stimulation of the nerve at various frequencies is illustrated in Fig. 1. In *A* stimuli were applied at 9 sec intervals, and each stimulus gave rise to a twitch-like movement of the hair. In this experiment, in which the nerve was stimulated at a distance of 20 mm from the muscle, the movement was recorded after a delay of 0.3 sec. When the stimulating electrode was located at this distance from the muscle the latency of the response

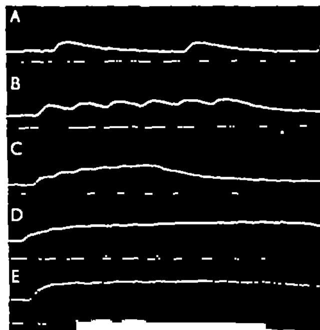


Fig 1 Pilomotor muscle contraction as reflected l the movement of single sinus hair l response to supramaximal stimulation of the nerve at various frequencies, i.e. *A* 0 11 *B* 0.4 *C* 0.7 *D* 1 1 *E* 7.0 stimuli per sec Time bar 2 sec

varied from 0 ? to 0 6 sec in different experiments. In that illustrated in *A* the peak of the contraction was attained in 0.8 sec (range in different experiments 0 6 to 1 0 sec) During the relaxation phase the hair slowly returned to resting position which in this experiment was reached in 6 sec.

In response to stimulation at frequencies higher than 0.2/sec summation occurred. The degree of summation increased with increasing rates of stimulation, as is shown in Fig 1 *B* and *C* in which the frequencies were 0.4 and 0.7 per sec respectively Single contractions could usually be followed up to a rate of one stimulus per sec, but on repetitive stimulation at higher frequencies (*D* and *E*) the contraction changed after an initial continuously rising phase to a steady plateau level The rate of rise of the initial phase and the amplitude of the plateau are higher i *E* (7 stimuli per sec) than in *D* (1 1 stimulus per sec) and thus increase with increasing stimulus frequency but no further rise in amplitude was observed at rates exceeding 10 per sec. The time required for the hair to return to resting position after cessation of the repetitive stimulation varied w dely but seemed to be longer after a long series of tetanic stimuli.

By introducing a metal microelectrode close to the hair it was possible simultaneously to record the contraction response and the electrical activ ty in the pilomotor musculature An experiment of this type is shown in Fig. 2, in which single (*A*) and repetitive (*B* and *C*) supramaximal stimuli were applied to the nerve 20 mm from the musculature In record *A* a slow slightly diphasic potential of comparatively low amplitude is initiated after a latency of 60 msec and 0.4 sec later a movement is recorded. On repetitive stimulation the potential changes in response to the second

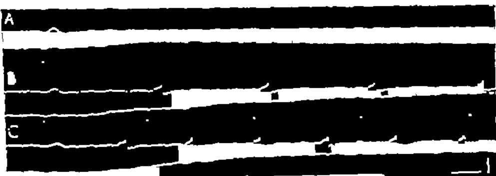


Fig. 2. Simultaneous recording of electrical activity (upper beam) and contraction (lower beam) of pilomotor muscles in response to supramaximal stimulation. *A*, single shock stimulation. *B* and *C*, repetitive stimulation at 0.7 and 1.1/sec respectively. Time bar 0.5 sec. Vertical bar 0.2 mV.

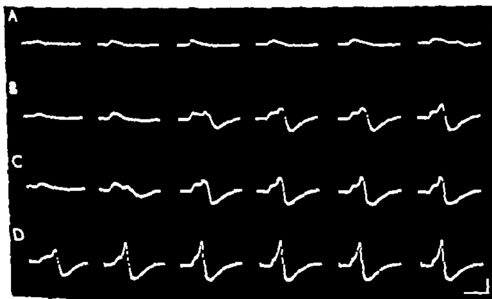


Fig. 3. Electrical responses of pilomotor muscles to repetitive stimulation rate of 0.5/sec in series of varying stimulus intensities, as: 4.5 V 1 msec. *B*, 4 V 1 msec. *C*, 3 V 1 msec. *D*, 10 V 2 msec. Stimulus applied at the beginning of each sweep. Time bar 0.2 sec. Vertical bar 0.- mV.

stimulus in a series with a frequency of 0.7/sec (*B*) d phasic pike potential appears, the amplitude of which is higher on the subsequent timuli. At this frequency ther is a noticeable increase in contraction w th each response. At a frequency just bove 1/sec (*C*) a similar facilitation of the initial responses occur but no single contractions can then be discerned

In order to study the two components of the electrical response more closely the number of activated pilomotor nerve fibers was aried by changing the stimulus

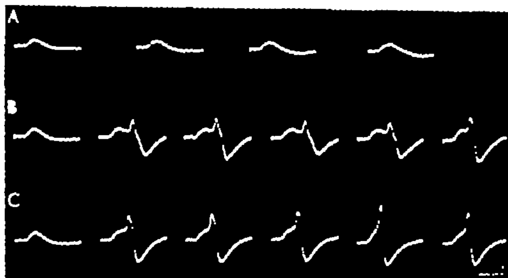


Fig. 4. Facilitation of the electrical response to repetitive stimulation of varying frequency rates, viz., *A* 0.1 *B* 0.4 *C* 0.7/sec. Supramaximal stimulation applied at the beginning of each sweep. Time bar 0.2 sec. Vertical bar 0.2 mV.

strength. Fig. 3 illustrates an experiment in which the nerve was stimulated once every two seconds in brief series. At a stimulus strength just above threshold (*A*) only a slow potential usually results the initial phase of which is positive. No complete spike is produced, but occasionally another component of low amplitude may appear after a long delay (the last record in *A*). The initial dominant phase of the slow potential has a duration of about 150 msec, and its rate of rise and amplitude increase on repetitive stimulation and when the stimulus strength is increased. In response to the stronger stimuli in *B*—*D* a spike may take off which is then of the same polarity as the slow potential. In *B* no spike is initiated until on the third stimulus, the following spikes gradually increase in amplitude while the latency to the point at which they take off from the slow potential is reduced. On a slight further increase in stimulus strength the second stimulus may produce a spike (*C*) and on supramaximal stimulation a spike may be initiated even by the first stimulus (*D*). As a rule, however, no spike occurred until at least two stimuli had been applied. After 4—6 stimuli the spike reached its final amplitude, the height of which depended on the stimulus strength. The amplitude varied somewhat from one stimulus to the other and on weak stimuli the spike sometimes failed to appear.

In other series of experiments the stimulus frequency was varied while the strength was kept constant. In Fig. 4 the nerve was stimulated at rates of 0.1, 0.4 and 0.7 stimuli per sec. At the lowest frequency only a slow potential occurred (*A*) which remained unchanged on repeated stimulation. At the higher frequencies used in *B* and *C* there was a gradual facilitation of the slow potential and a spike took off the amplitude of which increased with increasing stimulus frequency. The frequency



Fig. 5. Absence of any spikes during contraction induced by repetiti supramaximal stimulation at rate of 0.5/sec Time bar 0.5 sec Vertical bar 0.2 mV

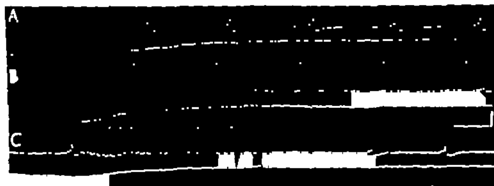


Fig. 6. Electrical activity (upper beam) and contraction (lower beam) in response to high-frequency stimulation. *A* 1/sec *B* 7/sec *C* t first 11/sec and then 1 1/sec. Time bar 0.5 sec. Vertical bar 0.2 mV

dependence of the spike varied in different experiments. As seen in Fig. 3 *D* a spike may take off even on a single shock. By contrast, Fig. 5 shows an experiment in which supramaximal repeated stimulation at a rate of 0.5/sec evoked only slow potential changes. It should be noted that each stimulation resulted in a contraction even though no spike was produced.

The effect of comparatively high tumulus frequencies on the acti ty of the pilomotor muscles is illustrated in Fig 6, in which th nerve was stimulated t fre quencies p to 11/sec. At a rate of one stimulus per sec (*A*) the potential at first changed as described above, and from the fourth stimulus the response remained unchanged. At rates of 7 and 11 stimuli per sec (*B* and first part of *C* respectively) the response changed so that from the third stimulus no spike occurred but only a slow potential change of gradually diminishing amplitude E en at a rate of 11 stimuli per sec each stimulus resulted in a response and even when no pikes were recorded the contraction was sustained In F'g 6 *C* after brief initial stimulation t 11/sec, the frequency was changed to 1 1/sec. and the electrical response was then once more gradually built up n the same manner as in *A*

In some experiments, the conduction eloc ty in the p lomotor nerv fibers under study was determined by placing th timulating electrode t two diff rent distances from the muscle (19 and 34 mm respectively The difference in latency of the slow potential changes thus observed corresponds to a conduction velocity of about 0.75 m/sec

Discussion

A pilomotor muscle contraction is but indirectly reflected in the movement of the sinus hair and a recording of this movement cannot thus be claimed to provide a fully accurate image of the contraction. Various factors may be involved in the transformation of a contraction into a hair movement, in the first place the turgor of the surrounding tissue and the state of tension of the skin. Rosenblueth (1937) meant that the tissue should offer a linear resistance and that the amplitude of the movement should thus be directly proportional to the amount of contraction, but no evidence was presented for this hypothesis. Since it is possible to record a pilo-erection during a contraction induced by a single shock, the resistance of the tissue is likely to be relatively small, and the hair movement should thus fairly closely reflect the time course and height of the contraction. No direct comparisons can however be made of the contraction amplitudes obtained in different experiments since the mechanical events may vary from one experiment to the other and since the recording of the movements is fully linear only over a very small area. The relatively wide differences in latency from the application of the stimulus to the first detectable movement (0.2 to 0.6 sec) may be an indication of variations in these mechanical events.

No dynamic recordings of movements of a single hair have previously been made, and no data are available on the contraction times of the pilomotor muscles. However Rosenblueth and del Pozo (1942) recorded the summed contraction of the pilomotor muscles of a number of hairs in the cat's tail, and an estimate of the contraction times can be made from some of their records. The value thus calculated amounts to 0.8 sec a figure which is of the same order of magnitude as those found in the present experiments.

The return of the hair to resting position is more dependent on the mechanical resistance of the surrounding tissue than is its elevation, and the time taken for the return is fairly independent of the relaxation of the muscle. In the course of a long tetanic contraction the tissue may adapt to the new position of the hair and these purely mechanical factors may explain why the hair is brought back more slowly after long series of stimulation at high frequencies. It cannot be excluded, however that the large number of impulses then set up in the nerve may release a large amount of transmitter which is accumulated. A similar after-effect during stimulation at high frequencies has been described in experiments on nictitating membrane (Rosenblueth 1932).

In *in vitro* studies of the pilomotor musculature in cat tail Hellman (1963 a, b) found that when using a temperature of 30° C repetitive stimulation at frequencies above 1/sec produced a fused tetanic response and in the present experiments (temperature 33—35° C) a fused response was observed when frequencies of 1 stimulus per sec or more were employed. The effect of temperature changes on the smooth muscles of the tactile sinus hair was not investigated, but Hellman found, when re-examining the tail pilomotor muscles at 20° C, that a fused response

could be obtained at or above frequencies of 0.13 c/s. It does not seem unlikely that the fusion frequency of the pilomotor muscles may be affected by temperature variations in the same manner as is striated muscle (Ritchie 1954)

In his *in vitro* experiments Hellman obtained maximal contraction amplitudes at stimulus frequencies of 10 to 15 per sec. Rosenblueth (1932) reported the same values from his experiments *i vivo* and also the movement of the tactile sinus hair attains its maximum amplitude at these frequency rates.

In the present experiments the electrical activity during contraction of the pilomotor muscles was recorded by an external electrode, and the potential change thus obtained should represent the sum of the ctivity in a number of cells around the electrode tip. It may seem surprising that a response of initial positive polarity could be recorded by this method. It has however been possible to record positive slow potential changes and spikes from visceral smooth muscle with glass capillaries of relatively large diameter so-called pressure electrodes (Bortoff 1961 Gillespie 1962) and in a comparative study of intra and extracellular electrodes on intestinal muscle Kobayashi, Prosser and Nagai (1967) found that also a metal microelectrode with a tip of 1 μ may act as a pressure electrode. Experiments on visceral smooth muscle have shown that extracell larly recorded potential changes may be difficult to interpret. According to Bortoff (1967) the rhythmic slow activity recorded from cat jejunum may represent a combination of a propagated slow wave and its associated field potential. Whether one or the other of these components will prevail depends on the magnitude of the shunting resistance around the electrode tip, and this in turn depends on the shape of the electrode. The studies of pressure electrodes were performed using visceral smooth muscle, the cells of which depolarize on mechanical deformation. The fact that the response recorded from th pilomotor muscles is positive suggests that cells in the close vicinity of the electrode tip may have been depolarized as a result of pressure or injury Electron microscopical studies have yielded results suggesting that the low resistance intercellular bridges present in, *e g* visceral smooth muscle may occur also in pilomotor muscles (Or fanos 1966) If this holds, then acti ity in neighboring non-injured cells may spread across these bridges and be recorded from the depolarized cells as a positive potential.

As appears from Fig. —4 at least two components may be discerned in the electrical acti ty during contraction of the pilomotor muscles, *vi*. a slow potential hange of low amplitude and a superimposed component of spike type. The general configuration of these potential changes has a remarkable similarity to the i tracellularly recorded activity in *g* the as deferens and in ascular smooth muscle. This activity consists of graded local responses. so-called junction potentials, and spikes (Burnstock and Holman 1961 Speden 1964) The slow potential change recorded from the pilomotor musculature was dependent both on the number of nerve fibers stimulated and on the stimul frequency The increase n amplitude in response to an increase in stimulus strength ma impl that progressively larger number of muscle cells are being act vated thus contributing to the total response obtained. However Burnstock and Holman (1966) demonstrated that junction

potentials recorded in single cells of the vas deferens in different species show a similar dependence on the stimulus intensity; this was interpreted as due to polyneuronal innervation, and it cannot be excluded that also the pilomotor muscle innervation may be of this type. The first slow potential changes in a series of repetitive stimulations gradually increase in amplitude and rate of rise. A similar facilitation of junction potentials on repetitive stimulation occurs in, *e.g.* the vas deferens (Burnstock and Holman 1961). For pilomotor muscles as for the vas deferens, the number of stimuli required before a spike develops is larger on submaximal than on supramaximal stimulation.

The amplitude of the spike potential increases with the stimulus strength and this is very likely due to activation of an increasing number of muscle cells. The facilitation of the spike on repetitive stimulation may be due to facilitated junction potentials that have reached threshold, thus giving rise to spikes in a successively larger number of cells. But an increase in spike amplitude may also result from an increasing synchronization of the discharges in different cells. A similar facilitation on repetitive stimulation of the pilomotor muscles of the cat's tail was described by Lambert and Rosenblueth (1935) and in studies of the electrogram of nictitating membrane Nyström (1962) found an increase in spike amplitude on repetitive low frequency stimulation. However, since these investigators stimulated preganglionic fibers the facilitation observed by them may not necessarily have occurred in the muscle.

A vertebrate smooth muscle contraction is almost invariably accompanied by the discharge of a propagated spike. Although in the present experiments a hair movement was sometimes recorded even though no spike could be observed, it is not warranted to draw the conclusion that a pilomotor muscle contraction can occur in the absence of a spike discharge in single cells, since a spike may have been set up in cells located so far from the electrode that their activity was not recorded. Tomita (1967) has shown that if the spike amplitude is not sufficiently high the propagation of the spike along cells and bundles of cells in the guinea-pig taenia coli may be decremental due to a low safety factor at the points where the different bundles branch. In an electron microscopical study of a cutaneous muscle—which is most likely to have been a pilomotor muscle—Orfanos (1966) found that the cells were unsmooth and branching. Also this type of smooth muscle may thus possess morphological properties which do not permit impulses to be conducted far enough to reach the electrode.

At high frequency rates the spike was substituted by a rhythmic oscillation of the same frequency as the stimulus. The same observation was made by Rosenblueth, Davis and Rempel (1936) on cat tail pilomotors and on nictitating membrane and in studies of this latter membrane Nyström (1962) found that the single potential changes begin to interfere with one another at stimulus frequencies above 3 or 4 per sec. On repetitive direct stimulation of the guinea pig taenia coli at frequencies up to 5 per sec, Tomita (1967) found the amplitude of the intracellularly recorded spike to be progressively reduced because the stimuli were applied during the re-

fractory period. These reduced spikes had a lowered ability to conduct along the bundle of cells due to the low safety factor at the branching points. A similar mechanism may explain why the spike of the pilomotor muscles is abolished at high stimulus frequencies. A reduced amplitude of the combined spike potential may however also be due to alternating discharges of small muscle units and an incomplete synchronization of the single cells, and this concept gains some support from the fact that the duration of the spike increases before it is abolished. It is of some interest to note that Gillespie (1962) in intracellular recordings from single smooth-muscle cells of rabbit colon, found that the spikes obtained in response to parasympathetic nerve stimulation became smaller at frequencies higher than 2/sec and were abolished when the frequency was still further increased. They were then substituted by a damped oscillation of the membrane potential which was gradually transformed into a steady state of depolarization. The muscle tension was then maximal and was maintained at this level although no spikes were recorded this was thought to illustrate that also in vertebrate smooth muscle a contraction may occur in the absence of a spike. Gillespie points out that the frequency at which this sustained depolarization begins to appear corresponds to the frequencies at which the slow potential changes (junction potentials) begin to overlap, which according to him may probably indicate an accumulation of transmitter Another finding in his experiments was that high stimulus frequencies showed a fatigue effect, in that the same low-frequency stimulation which before the tetanic series produced spike responses could later only evoke slow potential changes. As seen in Fig 6 C high-frequency stimulation of the pilomotor muscles may produce virtually the same effect.

Eccles (1935) found the conduction velocity in the cat's pilomotor nerve fibers from the superior cervical ganglion to be 1 7 to 5 m/sec, whereas Rosenblueth *et al* (1936) calculated the conduction velocity in the pilomotor nerves to the tail to be 1.5 to 4 m/sec. The value found in the present study 0 75 m/sec, is lower but nevertheless agrees well with determinations of the conduction velocities in other postganglionic sympathetic fibers (Kuriyama 1963)

Impulse conduction along the main nerve trunk accounts for about half of the interval between the application of stimulation and the onset of the slow potential (*cf* Results) thus leaving about 30 msec for impulse spread in fine nerve endings and for neuromuscular transmission. For cat tail pilomotors Rosenblueth *et al* (1936) found the corresponding value to be 40 msec. In the vas deferens the shortest latency on stimulation of intramural nerve fibers is 6 msec (Kuriyama 1963) a corresponding transmission time in the pilomotor muscles would leave most of the remaining time for impulse spread in the finest sympathetic terminal nerve branches in which the propagation velocity is known to be very low (Burnstock and Holman 1966)

References

Bortoff, A. Slow potential variations of small intestine. *Amer. J. Physiol.* 1961. *201*, 203—208.

Bortoff, A., Configuration of intestinal slow waves obtained by monopolar recording techniques. *Amer. J. Physiol.* 1967. *213*, 157—16..

Burn, J. H., E. H. Leach, M. J. Rand and J. W. Thompson. Peripheral effects of nicotine and acetylcholine resembling those of sympathetic stimulation. *J. Physiol.* (Lond.) 1959. *148*, 33 —55..

Burnstock, G. and M. E. Holman. The transmission of excitation from autonomic nerve to smooth muscle. *J. Physiol.* (Lond.) 1961. *155*, 115—133.

Burnstock, G. and M. E. Holman, Junction potentials at adrenergic synapses. *Pharmacol. Rev.* 1966. *18*, 481—493.

Eccles, J. C., The action potential of the superior cervical ganglion. *J. Physiol.* (Lond.) 1935. *85*, 179—206.

Fritz, F. Über einen Sinnesapparat am Unterarm der Katze nebst Bemerkungen über den Bau des Sinusbalges. *Z. wiss. Zool.* 1909. *92*, 291—305.

Fuxe, K. and B. Y. Nilsson, Mechanoreceptors and adrenergic nerve terminals. *Experientia* (Basel) 1965. *21*, 641—642.

Gillespie, J. S., The electrical and mechanical responses of intestinal smooth muscle cells to stimulation of their extrinsic parasympathetic nerves. *J. Physiol.* (Lond.) 1962. *162*, 76—92.

Hellmann, K., The isolated pilomotor muscles as an in vitro preparation. *J. Physiol.* (Lond.) 1963 a. *169*, 603—670.

Hellmann, K., The effect of temperature changes on the isolated pilomotor muscles. *J. Physiol.* (Lond.) 1963 b. *169*, 6.1—629.

Kobayashi, M., C. L. Prosser and T. Nagai, Electrical properties of intestinal muscle as measured intracellularly and extracellularly. *Amer. J. Physiol.* 1967. *13*, 275—286.

Kuriyama, H. Electrophysiological observations on the motor innervation of the smooth muscle cells in the guinea-pig vas deferens. *J. Physiol.* (Lond.) 1963. *169*, 13—228.

Lambert, E. F. and A. Rosenblueth. A further study of the electric responses of smooth muscle. *Amer. J. Physiol.* 1935. *114*, 147—159.

Nilsson, B. Y. and C. R. Skoglund. The tactile hairs on the cat foreleg. *Acta physiol. scand.* 1965. *65*, 364—369.

Nystrom, R. A., Nervous control of the cat nictitating membrane. *Amer. J. Physiol.* 1962. *202*, 849—855.

Orfanos, C. Elektronenmikroskopische Untersuchung glatter Haarmuskelfasern und ihrer Innervation. *Dermatologica* (Basel) 1966. *132*, 445—459.

Ritchie, J. M. The duration of the plateau of full activity in frog muscle. *J. Physiol.* (Lond.) 1954. *124*, 605—612.

Rosenblueth, A. The chemical mediation of autonomic nervous impulses as evidenced by summation of responses. *Amer. J. Physiol.* 1933. *102*, 1 —38.

Rosenblueth, A., H. Davis and B. Rempel. The physiological significance of the electric responses of smooth muscle. *Amer. J. Physiol.* 1936. *116*, 387—407.

Rosenblueth, A. and E. C. del Pozo, The electric responses of the tail pilomotors and nictitating membrane of the cat. *Amer. J. Physiol.* 194.. *137*, 763—79.

Speden, R. N. Electrical activity of single smooth muscle cells of the mesenteric artery produced by splanchnic nerve stimulation in the guinea pig. *Nature* (Lond.) 1964. *202*, 193—194.

Tomita, T. Spike propagation in the smooth muscle of the guinea-pig taenia coli. *J. Physiol.* (Lond.) 1967. *191*, 51 —527.

Wolbarsht, M. L. and H. G. Wagner. Glass-insulated platinum microelectrodes design and fabrication. In *M. d. al Electronics Proc. di gi of the fifth int. conference on medical electronics*. Liège July 22—26. 1963. p. 310—315.

Wolner, E. Versuche über die Innervation der Pilomotoren. *Naunyn-Schmiedeberg. Arch. exp. Path. Pharm.* 1965. *250*, 43 —450.

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Gastrin Like Activity in Different Parts of the Gastro-Intestinal Tract of the Cat

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Abstract

Emås, S and B. Fyrö *Gastrin-like activity in different parts of the gastro-intestinal tract of the cat* Acta physiol. scand. 1968. *74* 359—367

Mucosal specimens from different parts of the gastro-intestinal tract of cats were extracted for gastrin according to the method of Komarov. Extracts were also prepared from the mucosa of the antrum and proximal duodenum according to a modification of a method of Gregory and Tracy. The extracts were assayed for gastrin activity on nonanesthetized gastric fistula cats. Gastrin-like activity was found in the mucosa of the antrum and the proximal duodenum using either method of extraction. Gastrin-like activity was also found in most extracts from the cardiac region of the stomach. The gastrin-like activity of the extracts from the cardiac region and the proximal duodenum was 5—10 per cent of the antral activity. No gastrin-like activity was detected in the acid secreting part of the stomach, in the middle and distal parts of the duodenum, in the jejunum, colon or pancreas.

The hormone gastrin has been isolated from the antral mucosa of hog, man, dog and sheep and chemically characterized (Gregory and Tracy 1964, *Gregory et al.* 1964, Gregory, Tracy and Grossman 1966, *Gregory 1966*). Strong evidence has also been provided that Zollinger-Ellison tumors of the human pancreas contain gastrin (Gregory *et al.* 1967). No gastrin-like activity has been detected in the corpus region of the stomach. Studies on the distribution of gastrin-like activity in other parts of the gastro-intestinal tract have given contradictory results.

Gastrin-like activity has been detected in the duodenal mucosa of dog, hog, man and cat by some investigators (*Komarov 1938, 1942, Harper 1946, Lai 1964, Emås and Fyrö 1965*) but was not found in hog and dog by others (Uvnäs 1945 a, Gregory and Tracy 1961, Elliott *et al.* 1963). In jejunum, ileum and colon from man low gastrin-like activity was found when the extracts were assayed on anesthetized rats (Lai 1964), but no activity has been detected in extracts from these regions of cats, dogs or hogs when anesthetized cats were used as test animals (Komarov 1938, 1942, Uvnäs *1945*, Harper 1946).

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TABLE I Reports of gastrin-like activity in the gastro-intestinal tract apart from the antrum and Zollinger Ellison tumors of the pancreas.

Region	Gastrin-like activity	Species	Investigators
Cardia	−	cat	Lim 1922, Emås and Fyrö present study
	+	hog	Edkins 1905, 1906, Gregory and Tracy 1961
	0	hog	Uvnäs 1943.
Corpus	0	cat	Lim 1922, Uvnäs 1943, Emås and Fyrö present study
	0	dog	Komarov 1938, 1942, Uvnäs 1943, Elliot *et al.* 1963.
	0	hog	Edkins 1905, 1906, Uvnäs 1943, 1945 a, Harper 1946, Gregory and Tracy 1961
	0	man	Uvnäs 1945 b.
Duodenum	+	cat	Emås and Fyrö 1965, Fyrö 1967, Emås and Fyrö present study
	(+)	cat	Lim 1922, Uvnäs 1943.
	−	dog	Komarov 1938, 1942.
	0	dog	Uvnäs 1943, Elliott *et al.* 1963.
	+	hog	Harper 1946.
	0	hog	Uvnäs 1943, 1945 a, Gregory and Tracy 1961
	−	man	Lai 1964
	(+)	man	Uvnäs 1945 b.
Jejunum	0	cat	Lim 1922, Emås and Fyrö present study
	0	dog	Komarov 1938, 1942.
	+	man	Lai 1964
Ileum	0	cat	Lim 1922, Uvnäs 1943.
	0	dog	Uvnäs 1943.
	0	hog	Harper 1946.
	−	man	Lai 1964
Colon	0	cat	Uvnäs 1943, Emås and Fyrö present study
	0	dog	Uvnäs 1943.
	+	man	Lai 1964
Pancreas	0	cat	Emås and Fyrö present study
	0	dog	Elliott *et al.* 1963, Hallenbeck *et al.* 1963.
	0	hog	Hallenbeck *et al.* 1963.
	0	man	Hallenbeck *et al.* 1963

The contradictory reports of gastrin-like activity in parts of the gastro-intestinal tract, other than the antral mucosa and Zollinger Ellison tumors (Table I) may have several explanations. The distribution of gastrin-like activity may vary among species and different methods have been used for extraction of the gastrin-like activity and also for the assay of the extracts.

We have previously reported gastrin-like activity in the mucosa of the proximal

duodenum of cats and estimated the activity to be about one tenth of that in the antral mucosa (Emås and Fyrö 1965). The aim of the present investigation was to study further the distribution of gastrin-like activity in the gastro-intestinal tract of cats, using a standardized version (Emås and Fyrö 1964) of the method of Komarov (1938) for the extraction of gastrin. In addition, both the method of Komarov (1938) and a modified version of a method of Gregory and Tracy (1964) were used to prepare gastrin extracts from the mucosa of the antrum and proximal duodenum from the same cats. The study was carried out on one species, since all extracts were assayed for gastrin-like activity on nonanesthetized gastric fistula cats (Uvnäs and Emås 1961).

Methods

Healthy cats (1.8—4.0 kg) fasted for 24 hrs were used in the study. They were killed by an intraperitoneal injection of pentobarbital (300 mg/kg). The stomach, duodenum, pancreas and parts of the jejunum and colon were removed immediately after death.

Preparation of Mucosa Specimens

The stomach and duodenum were opened along the lesser curvature. At the gastro-oesophageal junction 1 cm of the gastric (cardiac) mucosa was stripped off from the whole circumference. A 1 cm strip of the corpus mucosa was taken 3—4 cm aboral to the cardiac region. A segment of the antral mucosa was stripped off 0.5—2.5 cm oral to the pyloric sphincter. In the duodenum, the mucosa from 4 successive 4 cm segments were stripped off 0.5—16.5 cm distal to the pyloric sphincter. In most experiments only the proximal segment was taken. When two different procedures for the extraction of gastrin were to be compared, each mucosa specimen from the antrum and from the proximal duodenal segment was divided along the greater curvature into two approximately equal halves. The halves were extracted by the different methods. A segment of the jejunal mucosa about 25—50 cm distal to the pyloric sphincter and a colonic segment about 5—10 cm distal to the ileo-caecal junction were taken. The mucus was gently scraped off the mucosa specimens which were then stored at −20° C.

Preparation of Gastrin Extract

Gastrin was extracted according to the method of Komarov (1938). This method involves boiling the mucosa in acid and precipitating the extract with trichloroacetic acid. After standardization (Emås and Fyrö 1964) this method has shown satisfactory reproducibility (Emås and Fyrö 1964, 1965). Each gastrin extract originates from the pooled mucosa specimens from the same part of the gastrointestinal tract, or from the pooled pancreas, of two cats. A series of 3—6 extracts from the same two cats was prepared simultaneously. In every series one extract was derived from the antral mucosa. Care was taken to treat all batches identically.

Four extracts were prepared according to the Komarov method from each of the corpus of the stomach, the jejunum, colon and pancreas, and 6 extracts from the cardiac region of the stomach. Nine extracts were prepared from the proximal duodenal segment and 2 extracts from each of the 3 successive duodenal segments (Table II).

To compare two different procedures for the extraction of gastrin, 4 halves of antral mucosa from 4 cats were pooled and extracted by the method of Komarov (1938) and the other pool of 4 antral halves was extracted by a simplified version (Andersson and Nilsson 1968) of the method of Gregory and Tracy (1964). From the same 4 cats the proximal duodenal segment was collected and treated in the same way as the antral mucosa specimens. Two antral and two duodenal extracts were prepared simultaneously according to both methods.

The simplified method of Gregory and Tracy involved boiling the minced mucosa specimens (about 2 g) in 50 ml of distilled water for 1—2 min. The mixture was homogenized (Ultra-Turrax homogenizer 20,000 rpm) for 1 min and then boiled for another 10 min. After cooling, the suspension was filtered through paper (Munktell No 3) and the filtrate was centrifuged. The precipitate was discarded and 0.5 g of diethylaminoethylcellulose "floc" (Whatman DE 50) was added to the supernatant. The suspension was adjusted to pH 5, stirred for 3 hrs, and then centrifuged. The precipitate was washed twice by stirring with 100 ml of distilled water for 10 min and centrifuging. The fraction containing the gastrin-like

activity was eluated by treating the floc with 0.1 N NaOH (40 ml/g mucosa). The floc was stirred with 1/4 of the total volume of 0.1 N NaOH for 10 min and then centrifuged. This procedure was repeated 3 times. The 4 supernatants were mixed, filtered twice, adjusted to pH with HCl and then stored at −20 C until assayed.

Ten gastrin extracts were prepared from pooled antral mucosa specimens according to each method, and similarly 10 extracts were prepared according to each method from pooled specimens of the mucosa of the proximal duodenum (Table II). Fi gastrin extracts made by each method were randomly selected and assayed on guinea-pig ileum for histamine activity. No histamine was detectable in any extract (<0.05 µg of histamine dihydrochloride per mg of the Komarov extract or per ml of the Gregory Tracy extract).

Assay of Gastrin Activity

The acid secretory activity of each gastrin extract was assayed on non-anaesthetized gastric fistula cats using histamine as the reference standard, as described by Uvnäs and Emås (1961). All extracts from a series were assayed on the same 2—4 animals, the number of animals depending on the amount of extract available. In the experiments comparing the two extraction methods, the antral and duodenal gastrin extracts derived from the same animals were assayed on the same 2 cats.

The dose of the antral gastrin extracts prepared according to the Komarov method was 0.5 mg/kg and according to the Gregory and Tracy method 0.25 or 0.5 ml/kg. The dose of extracts from other tissues was 4 mg/kg and 2 or 4 ml/kg, respectively. One mg of the Komarov gastrin extracts from antrum and duodenum was derived from 14.1 ± 0.7 (mean ± S.E., n = 20) mg of mucosa and 1 ml of the Gregory and Tracy extracts from 25 mg of mucosa (= 20). If 4 mg/kg was insufficient to produce a response equal to or greater than the response to the low reference dose of histamine the gastrin-like activity of the extract was considered to be below the measurable level of the assay method.

The gastrin-like activity was expressed in histamine units (HU) (Uvnäs and Emås 1961) per mg or ml extract and in HU per g of tissue. The activity was also calculated as per centage of the antral activity in each series.

Determination of the Effect of Cholecystokinin-Pancreozymin Gastric Acid Secretion

these experiments highly purified preparation of cholecystokinin-pancreozymin kindly plied by Professor Jorpes) was used. It contained 1500 Ivy dog units of cholecystokinin and 000 Crick Harper and Raper units of pancreozymin activity per mg. The stimulatory effect on gastric acid secretion of this preparation was tested in 4 experiments on each of 6 gastric fistula cats Emås 1960 I 2 expts the cats were given 0.25 0.5, 1, 2 and 4 Crick Harper and Raper units/kg/hr and in 2 expts 4 8, 16 and 32 units/kg/hr. Each dose was given as continuous infusion during consecutive hours. The experiment started with the smallest dose. The dose was then doubled each hour.

In all experiments the gastric secretion was collected during 15 min periods, the volume was measured and the acidity determined by titration with 0.01 NaOH, using phenolphthalein as indicator.

In constructing the dose-response curves (Fig. 1) the response is defined as the mean output acid per 15 min calculated from the last two 15 min periods at each dose level.

Results

Gastrin-Like Activity of the Antral and Proximal Duodenal Mucosa

The yield of 19 antral extracts prepared according to the Komarov method was 75.2 ± 3.0 (mean ± S.E.) mg per g frozen mucosa. The secretory activity amounted to 21.7 ± 2.2 HU per mg extract and to 1590 ± 170 HU per g mucosa. The mean yield of duodenal extracts in the same series of extractions was 75.4 ± 3.0 (n = 19) mg per g mucosa. The mean activity for 17 of the duodenal extracts was 1.4 ± 0.2 HU per mg extract and 103 ± 14 HU per g mucosa. The activity per g mucosa was 8 per cent of the corresponding antral gastrin activity (Table II). Th activity of duodenal extracts was too low to be detected.

Microscopical examination of the proximal border of 5 duodenal mucosa specimens showed that no antral mucosa was present.

TABLE II Number of extracts and gastrin-like activity in different parts of the gastro-intestinal tract of cats in the present study

Method for extraction and region	Number of extracts Total	Activ	Mean gastrin-lik ctivity of activ extracts as per centage of tral gastrin ctivi y
Komarov method			
Antrum	19	19	100
Cardia	6	4	5
Corpus	4	—	—
Duodenum (0.5—4.5 cm)	19	17	8
Duodenum (4.5—8.5 cm)	2	1	10
Duodenum (8.5—12.5 cm)	2	—	—
Duodenum (12.5—16.5 cm)	2	—	—
Jejunum	4	—	—
Colon	4	—	—
Pancreas	4	—	—
Extracts from the same cats prepared by two methods			
Komarov method			
Antrum	10	10	100
Duodenum (0.5—4.5 cm)	10	10	5
Gregory Tracy method			
Antrum	10	10	132*
Duodenum (0.5—4.5 cm)	10	10	5

distance from pylorus.
activity in per cen of corresponding tral activity according to the method of Komarov

Gastrin Like A t ty Different S gments f th D od nal Mu sa
In 2 series, extracts were prepared from 4 duodenal segments by the Komarov method The me n activity per g mucosa in the proximal duodenal segment amounted to 152 HU or 12 per cent of the activity of antral mucosa. The activ ty in the second segment was as high as in the proximal segment in one series and below the measurable level in the other In the 3rd and 4th segments th acti t w too low to be measured (T bl II)

Gastrin Lik ict ty in other Pa t f th Gast o-I te t al T t
In extracts from the mucosa of the cardia region measurabl gastrin-like acti ty was found in 4 of 6 extracts (Table II) The mean activ ty of the 4 extracts amounted to 88 ± 1 HU per g mucosa, or 5 per cent of the correspond ng tral activ ty In

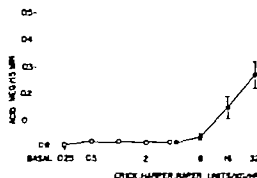


Fig 1 Dose response curves for gastric acid secretion in response t cholecystokinin-pancreozymin. Each curv is the mean of 2 ex periments on ach of 6 cats. The ertical bars represent S.E. of mean.

the 4 extracts from the mucosa of the corpus of the stomach, from the jejunal and colonic mucosa and from the pancreas, respectivel no gastrin-like activity was found.

Gastrin Like Activity in th Ant al and Proximal Duodenal Mucosa According to two Ext action Methods

Using the Komarov method for extraction, the antral gastrin activity was 1550 ± 210 (mean ± S E n = 10) HU per g mucosa, using the Gregory Tracy method it was 1930 ± 310 (n = 10) HU per g mucosa. The mean values for d odenal gastrin-like , were 75 ± 15 and 78 ± 24 HU per g mucosa, respecti ely or 5 and 4 per cent the corresponding antral activity (Table II)

According to current methods for analysis of variance (Snedecor 1956) there was no statistically significant difference ($p > 0.05$) between the gastrin activity of the antral extracts prepared by the two different methods. The same was true for the duodenal extracts prepared by the two methods.

Effect of Chol cystokin n-Pancreozymin on Gastric Acid Secretion

Cholecystokinin-pancreozymin was given as 1-hr infusions in doses of 0.25—32 Crick, Harper and Raper units/kg/hr Significant acid responses were obtained to doses of 16 and 32 units/kg/hr (Fig 1)

Discussion

The present results ha e demonstrated gastrin-like activity in the mucosa of the antrum and proximal duodenum of the cat. Low gastrin-like activ ty was also detected in 4 of 6 extracts from the cardiac region of the stomach. The activity found in the cardiac and duodenal mucosa was 5—10 per cent of that in the antral mucosa. No gastrin-like activ t was detected in the acid secreting part of the stomach, in the middle and distal parts of the duodenum, in the jejunum, colon or pancreas.

Low gastrin-like activity in th cardiac region of the cat is in agreement with the findings of Gregory and Tracy 1961 in the hog Earlier Edkins (1905 1906) and

Lim (1922) found gastrin-like activity in this region in hog and cat, but the significance of their findings has been questioned, since their preparations also contained histamine. It has been claimed that gastrin-like activity is present in the jejunum and colon in man (Lai 1964) but no such activity has ever been detected in the dog, cat or hog (Komarov 1938 1942, Uvnäs 1945 Harper 1946) In the corpus of the stomach (Komarov 1938, 1942, Uvnäs 1945 1945 a, b Harper 1946 Gregory and Tracy 1961) and in the normal pancreas (Elliott *et al* 1963 Hallenbeck, Code and McIlrath 1963 Lai 1964) no gastrin-like activity has been detected in any species investigated (Table I)

In the duodenal mucosa, gastrin-like activity has been found in cats, dogs and hogs by some investigators (Komarov 1938 1942, Harper 1946, Emås and Fyrö 1965, Fyrö 1967) but others have reported no activity in dogs and hogs (Uvnäs 1945, 1945 a, Gregory and Tracy 1961 Elliott *et al.* 1963) Slight activity was reported in 1 of 4 extracts from the duodenum of cats by Uvnäs (1945)

An extract of hog duodenal mucosa made by the method of Gregory and Tracy (1961) was very active when assayed on the anesthetized rat but showed no gastrin-like activity when given to a conscious dog (Lai 1964) This is difficult to explain unless rats are more sensitive to gastrin-like activity from hog duodenal mucosa than dogs are. Lai (1964) has also reported gastrin-like activity in duodenal extracts from human subjects assayed on anesthetized rats. In the present study the extracts obtained from the proximal duodenum of cats were active when assayed on the same species.

Significant gastrin-like activity in the duodenum was found only in its proximal part in the present study The failure of some authors to demonstrate any duodenal gastrin-like activity may be due to the fact that the extracts were prepared from the whole length of the duodenum, in which case the activity in the extracts may have been too low to detect. Other reasons may be that the duodenal gastrin-like activity varies among species and/or that some methods are unsuitable for extracting the low level of activity present in the duodenum.

In this study the antral and duodenal gastrin-like activity was estimated after using both the method of Komarov (1938) and that of Gregory and Tracy (1964) for extraction. The figures for the activity are comparable, since the mucosa specimens extracted by the two methods were collected from the same animals. Using either method, gastrin-like activity was detected in the antrum as well as in the proximal duodenum. Furthermore the gastrin-like activity obtained by the two methods was very similar

In the duodenal extracts gastro-intestinal hormones other than gastrin, influencing gastric acid secretion, might also be present Secretin and cholecystokinin-pancreozymin are known to inhibit gastrin induced gastric acid secretion in dogs (Gillespie and Grossman 1964 Brown and Magee 1967) and if present in our extracts they could reduce the stimulatory effect of duodenal gastrin On the other hand, it has also been reported that cholecystokinin-pancreozymin stimulates gastric acid secretion in dogs (Preshaw and Grossman 1965 a, b Murat and White 1966, Magee and Naka

mura 1966). In the present study only high doses of cholecystokinin-pancreozymin stimulated gastric acid secretion in cats. It seems, therefore, unlikely that the duodenal gastrin-like activity is due only to the presence of cholecystokinin pancreozymin in the extracts.

Whether the gastrin-like activity in the duodenal mucosa is due to the presence of gastrin or a factor related to gastrin, remains to be elucidated. In addition, the presence of inhibitory factors, masking some gastrin-like activity cannot be eliminated.

Earlier observations that both duodenal and antral gastrin like activity is reduced by reserpine in nonvagotomized but not in vagotomized cats (Emås and Fyrö 1965) and by vagal stimulation (Fyrö 1967) suggest a vagal release of the duodenal gastrin like factor as well as of the antral gastrin.

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References

Andersson, S. and G. N[illegible]. Levels of acidity in duodenal bulb inhibiting gastric acid responses to exogenous gastri[illegible]. *Act[illegible] physiol. sc[illegible]*. To be published.
[illegible] J. C. and D. F. Magee. Inhibitory action of [illegible]holecystokinin on acid secretion from [illegible]denhain pouches induced by endogenous gastrin. *Gut* 1967. *8*. 29—31.
[illegible] J. S. On the [illegible]hemical mechanism of gastric secretion. *P[illegible] roc[illegible]* [illegible] *Soc. B* 1905. *76*. 376.
[illegible] J. S. The chemical mechanism of gastric secretion. *J. Physiol.* (Lond.) 1906. *34*. 133—144.
Elliott, D. W., G. L. Ead[illegible], H. E. Knobbenschild, G. N. Grant, J. T. Gos[illegible] and R. M. Zollinger. Relation of antrum t[illegible] pancreatic-induced gastric hypersecretion. *S[illegible]gery* 1963. *54*. 9—18.
Emås, S. Gastric sec[illegible]tory [illegible]esponses to repeated intravenous infusions of histamine [illegible]nd gastrin in nonanesthetized and anesthetized gastric fistula cats. *Gast[illegible]enterology* 1960. *39*. 771—782.
Emås, S. and B. Fyr[illegible]. A[illegible]al gastrin acti[illegible]ity in duodenal and gastric ulcers. *Gast[illegible] [illegible]terology* 1964. *46*. 1—7.
Emå[illegible] S. and B. F[illegible]. Vagal release of gastri[illegible] in cats following reserpine. *A[illegible] physiol. sc[illegible]* 1965. *63*. 358—369.
Fyrö, B., Reduction of [illegible]tral [illegible]nd duodenal gastri[illegible] activity by [illegible]lectrical [illegible]gal stimulation. *Act[illegible] physiol. scand.* 1967. *71*. 334—340.
Gillespie, J. E. and M. I. Gro[illegible]. Inhibitory effect of secretin and [illegible]holecystokinin on Heidenhain pouch [illegible]esponses t[illegible] gastrin extract and histamine. *Gut* 1964. *5*. 342—345.
Gregory, R. A. Memorial le[illegible]. The [illegible]solation and chemistry of gastrin. *Gast[illegible]enterology* 1966. *51*. 953—959.
Gregory, R. A., M. I. Gro[illegible], H. J. T[illegible] and P. H. Bar[illegible]. N[illegible]re of the gastric secretagogue in Zollinger-Ellison [illegible]. *Lan[illegible]* 1967. *II*. 543—544.
Gregory, H., P. M. Hardy, D. S. Jon[illegible], G. W. K[illegible] and R. C. Sheppard. The antral hormone gastrin. Structur[illegible] of [illegible]. *Nat[illegible]* (Lond.) 1964. *204*. 931—933.
Gregory, R. A. and H. J. Tr[illegible]. The preparation and properties of gastrin. *J. Physiol.* (Lond.) 1961. *156*. 523—543.
Gregory, R. A. and H. J. Trac[illegible]. The constitution and properties of tw[illegible] gastrins extracted from hog antral mucosa. *Gut* 1964. *5*. 103—117.
Gregory, R. A., H. J. Tracy and M. I. Gro[illegible]. Isolation of tw[illegible] gastrins from human antral mucosa. *Nat[illegible]* (Lond.) 1966. *209*. 583.
Hallenbeck, G. A., C. F. Code and D. C. M[illegible]. Absence of demonstrable gastric secretagogue in normal pancreatic ti[illegible]. *Gast[illegible]enterology* 1963. *44*. 627—630.

HARPER, A. A., The effect of extracts of gastric and intestinal mucosa on the secretion of HCl by the cat stomach. *J Physiol.* (Lond.) 1946. *105* 31 P
KOMAROV S. A., Gastrin. *Proc Soc exp Biol.* (N Y) 1938. *38* 514—516.
KOMAROV S. A., Studies on gastrin. — II. Physiological properties of the specific gastric secretagogue of the pyloric mucous membrane. *Rev canad. Biol.* 1942. *1* 377—401
LAI, K. S Studies on gastrin. — II Quantitative study of the distribution of gastrin-like activity along the gut. *Gut* 1964 *5* 334—336.
LIM, R. K. S The question of a gastric hormone. *Q. Jl exp. Physiol* 1922. *13* 79—103
MAGEE, D F and M. NAKAMURA, Action of pancreozymin preparations on gastric secretion. *Nature* (Lond.) 1966. *212* 1487—1488.
MURAT J E. and T T WHITE, Stimulation of gastric secretion by commercial cholecystokinin extracts. *Proc Soc exp Biol.* (N Y) 1966. *123* 593—594
PRESHAW R. M. and M. I. GROSSMAN Stimulation of pancreatic secretion by extract of the pyloric gland area of the stomach. *Gastroenterology* 1965 *48* 36—44
PRESHAW R. M and M. I GROSSMAN Comparison of subcutaneous and intravenous administration of pancreatic stimulants. *Amer J Physiol.* 1965 *209* 803—810.
SNEDECOR, G. W *Statistical Methods* Ed. 5. Iowa State College Press. Ames. Iowa 1956
UVNÄS, B The secretory excitant from the pyloric mucosa. *Acta physiol. scand.* 1943 *6* 97—107
UVNÄS, B Further attempts to isolate a gastric secretory excitant from the pyloric mucosa of pigs. *Acta physiol. scand.* 1945 a. *9* 296—305.
UVNÄS, B The presence of a gastric secretory excitant in the human gastric and duodenal mucosa. *Acta physiol. scand* 1945 b. *10* 97—101
UVNÄS, B. and S. EMÅS, A method for biologic assay of gastrin. *Gastroenterology* 1961 *40* 644—648.

Discussion

Triton caused progressive increase in the amounts of cholesterol and esterified fatty acids in the plasma, as has also been noted in previous studies (Byers and Friedman 1960 Kramer and Gioia 1963) Janicki *et al* (1962) observed that triglycerides were bound to the plasma protein and they attributed the lipemia to inhibited activation of lipoprotein lipase

When the rat's heart is perfused with histamine liberator (compound 48/80) a lipase activator is detached probably from its mast cells, which has the same efficiency as that obtained by heparin perfusion (Ho Aktin and Meng 1966) During anaphylactic shock there occurs destruction of mast cells in the mucosa of connective tissue but not of the gastrointestinal tract, and inhibition of blood coagulation (Wilander 1958) On the other hand, endocrine effects are above all, reflected in the mast cells of the gastrointestinal mucosa (Räsänen 1967) Their degranulation results in alimentary lipemia and increase of cholesterol in the plasma (Haikonen and Räsänen 1963)

Triton caused increase in number of the mast cells in the gastric and jejunal mucosa in the present study The abundance and wholeness of the mast cell granules, above all, gives the impression that heparin liberation of these cells has probably been inhibited during Triton effect and this in turn is reflected by an inhibition of lipoprotein lipase activity and possibly accelerates the conversion of fatty acids into the protein-bound form (Janicki *et al* 1962)

Heparin seems to have a permissive effect on lipolysis (Menge and Elgren 1963) e elevated accumulations of esterified fatty acids in the plasma indicate that Triton probabl inhibits the heparin liberation during endogenous lipemia On the other hand the observation has been made that lipemia produced by exogenous fats seems to cause mobilization of endogenous heparin (Engelberg 1958)

Under the effect of growth hormone the mast cells of the gastric mucosa increase in number (Räsänen 1960 although the simultaneous degranulating gluc corticoid effect is permissive (Räsänen 1961) The mobilization of polysaccharides in the mucosal mast cells is thought to be relatively inhibited under growth hormone effect, which at the same time causes increase in the concentration of free fatty acid in dog blood (Winkler *et al* 1964 The opposite phenomenon, exhaustion of the polysaccharide supply in the mast cell of the gastrointestinal mucosa in reserpinized rats (Raslnen and Taskinen 1966 (or probably results in inhibition of heparin mobilization and in lipemia Abbound Wendling and Eckstein 1965 Friedman and Byers 1967) as occurs after degranulation effected with glucocorticoids (Haikonen and Räsänen 1965 Friedman *et al* 1965)

The hypoproteinaemia which ensues during Triton effect may be due to increased protein consumption when the production of energy by fats is inhibited, or when parts of the amino acid are possibl transformed int fatty acids

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References

ARMOCIDO F M M. C. WERDLING and J W ECKSTEIN, Effect of norepinephrine on plasma free fatty acids in dogs treated with reserpine. *Amer J Physiol.* 1963 *205* 57—59

BIANCHI, P Study of heparinoid of the duodenal mucosa. *Boll. Soc ital. Biol. sper* 1958. *34* 685—688.

BYERS, S. O and M. FRIEDMAN, Site of origin of plasma triglyceride *Amer J Physiol* 1960 *198* 629—631

CASTONE, A V RULLI and B. ROSSI, Antilipemic activity of an extract of hog gastric mucosa. *Circulat. Res* 1959 7 791—295

DUTTON G J and I H. S STEVENSON, Synthesis of glucuronides and of urine diphosphate glucuronic acid in kidney cortex and gastric mucosa. *Biochim. Biophys. Act* (Amst.) 1959 *31* 568.

ENGELBERG, H, Plasma heparin levels in man following intravenous fat emulsion. *Proc Soc exp. Biol.* (N Y) 1958. *97* 304—308

FRIEDMAN, M. J J VAN DEN BOSCH, S. O BYERS and S S GEORGE, Effects of cortisone on lipid and cholesterol metabolism in the rabbit and rat. *Amer J Physiol.* 1965 *208* 91—105.

FRIEDMAN, M. J and S. O BYERS, Epinephrine-induced reduction of postprandial lipemia in the rat. *Amer J Physiol.* 1967 *213* 829—836.

HAKONEN, M. and T RÄSÄNEN, The serum cholesterol level and mucosal mast cell count in the gastrointestinal tract of rats in dexamethasone-shock. *Act physiol. scand.* 1963 *59* 478—485.

HENRY, R. J C. SOBEL and S. BERMAN, Interferences with Biuret method for serum proteins. *Anal. Chem.* 1957 *29* 1491—1495.

HO, R. J E. ARTIN and H. C. MENG, Lipase release induced by Compound 48/80 in rat and perfused rat heart. *Amer J Physiol.* 1966. *210* 299—304

JUDGE, B W W V C. LEAHY TH. F MCNEALE and S A ARON, Effect of Triton WR 1339 on absorption and fate of J^{131} oleic acid in normal guinea pigs *Amer J Physiol.* 1962. *202* 367—369

KRAMER, M. and A. GIOIA, Action of Triton WR 1339 on the intestinal absorption of fat. *Proc First int Pharm. Meet* 1963. *2* 183—185

MARKS, I J S. BANK, L. H KRUT and B. BRONTE-STEWART Gastric secretion and alimentary lipaemia in ischaemic heart-disease *La et* 1962. *2* 1068—1072.

MENG, H G. and B. EDGREN, Source of plasma free fatty acids in dogs receiving fat emulsion and heparin. *Amer J Physiol.* 1963 *204* 691—695.

PEARSON S., S STERN and T H. MCGAVACK, A rapid accurate method for the determination of total cholesterol in serum. *Anal Chem.* 1953 *25* 813

RÄSÄNEN T Mucosal mast cells of rat stomach influence of ACTH, cortisone and growth hormone. *Gastroenterology* 1960. *38* 70.

RÄSÄNEN T, Effect of prednisolone and deoxycorticosterone, administered separately and with growth hormone, on the mucosal mast cells and tissue eosinophils of rat stomach wall. *Gastroenterology* 1961 *40* 232—234

RÄSÄNEN T Different hormonal responses of mast cells in gastric mucosa and dermis in the rat. *Scand. J Gastroent* 1967 *2* 118—123

RÄSÄNEN T and E TASKINEN Reserpine and gastrointestinal mucosal mast cells. *Act physiol. scand* 1966. *68* 360—364

SMITH, H and R. C. GALLOP The acid polysaccharides of hog gastric mucosa *Biochem. J* 1953. *53* 666.

STERN I and B. SHAPIRO *Bestimmung der Fettsäuren im Blut* I Medizinische Chemie 3 Aufl eds. Karl Hinsberg und Konrad Lang Urban & Schwarzenberg, München 1957 pp 447—448.

TERHO T K. HARTIALA and J HÄKKINEN Infra-red spectroscopic investigation of new acid mucopolysaccharide isolated from human gastric juice *Nat* Lond 1966 *211* 198—199

WILANDER, O Studien über Heparin. *Skand Arch Physiol* 1938 *81* Suppl 15

WINKLER, B R W STEELE, N ALTSZULER and R C DE BODO Effect of growth hormone on free fatty acid metabolism. *Amer J Physiol* 1964 *206* 1 4—1 8

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Elasticity and Tensile Strength of the Anterior Cruciate Ligament in Rabbits as Influenced by Training

By

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Abstract

Viidik, A. *Elasticity and tensile strength of the anterior cruciate ligament in rabbits as influenced by training* Acta physiol. scand. 1968 74 372—380

The effects of training in running machine were studied on nineteen rabbits while seventeen of the same stock were used as controls. The rabbits were the start of th training period bout three months old and the mechanical ests were performed forty weeks later Tensile strength and elasticity tests were performed on erior crucia ligament from their knee joints ing the femur and the tibia as fixture points. I is concluded ha the weakest point in the system of tibia-igamentum cruciatum erius—femur is one of the bony attachments and most often the tibial e. The energy required to break the system is increased by training and so are the maximum load and the deformation t that point All these phenomena can be accounted for by strengthening of he bones. The ela ticity tests, however show larger load-r laxation phenomenon in he ligaments from the trained animals and it occurs w th higher speed, indicating altered viscous properties I is concluded ha these cha ges must be qualitativ changes occurring in the ligament us tissue

Knee joint ligaments ha e been used by several investigators as con enient test specimen they onsist of well-defined parallel-fibred bundles of collagenous tissue with insertions nt the tibia and the femur which can easily be fastened into material te ting machines w thout l pping or jaw breaks Some investig tors have used the anterior ru iat ligament e g Annovazzi 1928, Smith 1954 Viidik 1965, 1966 while others have used tructures like the collateral ligaments (Annovazzi 1928 Schild et al 1967 T pton t al 196 Differing results on th exten il ility and elasticity of this type of tis ue ha e been eported While Annovazzi 1928) and Gratz 1931 found it elasti th n ertain limits Stucke 1950 reported an imperfection Smith 1954 laimed that t wa possible to maintain la ticity after short submaximum load but Rigby t al 1959 found a slight permanent di tension in the first testing cycle which they alled onditioning" after which the elasticity was perfect. W th an apparatus on tructed t prevent back lash and perm t accurat readings of small load o deform tion hanges Viidik 1966 found the anterior cruciate ligament of rabbits t b mperfectl elast even t moderate load and to possess significant viscous properties The elemen t of pla ticity was m st evident in

the first loading cycle. There was a clear difference between the slope (corresponding to the elastic stiffness) of the first loading cycle and the following ones. In the second and third loading cycle a little more deformation was required to achieve the same load as in the first cycle. No difference was found when the load was one tenth or one third of the failure load, if the calculations were made in per cent. The same was the case with the load relaxation phenomenon if the deformation was kept constant. If the resting period between cycles exceeded 10 min, it did not affect the results.

The morphological reaction of collagenous tissue in the locomotor system to increased functional demands was studied by Ingelmark (1945) who found that animals trained at a very early age enlarged the cross-sectional areas of their Achilles tendons and gastrocnemius muscles but only of the muscles when the training was performed on older animals (Ingelmark 1948). Training made medium sized tendons stronger when tested alone even when no quantitative changes occurred (Viidik 1968 b) but no definite conclusions could be made on the whole functional system consisting of calcaneus-tendo Achilles-m. gastrocnemius-femur as the intra-group variation was considerable in this study (Viidik 1968 a). The literature on how training affects different joint components is scarce and studies have been made from a morphological point of view and mostly on joint cartilage. The present study was undertaken to investigate how training in a running machine influences the elasticity and tensile strength of the anterior cruciate ligament and its attachments to the femur and the tibia in the knee joint of rabbits.

Materials and testing methods

19 male white rabbits were trained 3 times a day every working day (usually 5 days a week) for 40 weeks in a running machine. When the training started they were about 3 months old weighing 2.42 ± 0.05 kg, and skeletally immature. The training time and velocity were adjusted to the actual maximum capacity of the rabbits. The mechanical testing was performed after about 40 weeks of training. Then they were skeletally mature in their hind limbs (the criterion that the distal femoral and proximal tibial epiphyses should be calcified was adopted) and weighed 3.38 ± 0.13 kg. The rabbits covered a distance of over 100 km during the training which is described in detail elsewhere (Viidik 1968). Seventeen rabbits of the same stock were kept as controls for the same period of time and were permitted to move freely in the same type of standard cages (floor 69 × 45 cm) as the rabbits subjected to training. They weighed at the time of mechanical testing 3.61 ± 0.14 kg; the difference between these rabbits and the trained ones is not statistically significant.

The rabbits were sacrificed as described by Viidik (1968) and the tests on the calcaneus-Achilles-tendon-gastrocnemius muscle-femur complexes were performed. Care was taken to avoid exposure of the joint cavity and drying of the intra-articular structures. The tensile strength and elasticity testing was performed afterwards within two hours of death, a period with no influence on the tensile strength properties or histological picture (Viidik et al. 1965) or on the viscous properties (Viidik 1966) of the ligaments. On 4 of the rabbits in each group tensile strength tests were performed on both knees. On the remaining rabbits a tensile strength test was performed on one knee and elasticity test on the other, the selection of knees being at random.

For both types of experiments the femur and the tibia were severed at appropriate distances from the knee joint and all structures except the anterior cruciate ligament were removed. Then the femur and the tibia were fastened in contour-shaped clamps that prevented slipping of the bone ends without fracturing them. The clamps were movable in all planes but no rotatory motion was possible between the longitudinal axes through the bones.

This is important as the ligament is twisted which gives the impression that the tibia is rotated medially 90° from the femur. A rotatory movement would have permitted the ligament to untwist and giving false elongation reading.

The specimen is shown schematically in Fig. 1 where the solid lines indicate the starting position with no load applied. The dotted lines indicate the load P and the corresponding elongation of ΔL

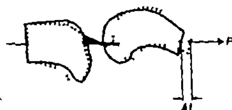


Fig. 1 A schematic picture of the test specimen. From th left, femur t the fixed end of the system, the anterior cruciate ligament nd tibia. The solid lines indicat the system before loading The dott ed lines indicate the configuration when the load of P has been applied resulting in the deforma- tion of Δl, the ligament being the only extensible structure

Th elongation of the system is in the ligament as it is the only extensible structure The t links permit th specimen change the longitudinal axes through the femur and the tibia slightly owing t the fact that the ligament is in an blique position. Therefore the true force acting on the ligament will be th measured force times factor greater th n unity and the true elongation of the ligament w ll be the measured elongation times factor less than unity As the specimens were of fairly uniform ppearance the multiplying factors were assumed constant between specimens at corresponding stages of th tests.

For the tensile strength experiments the clamps were fastened t to material testing machine. The moving end of this was opera ed by hydraulic system powered by an electric motor applying the deformation t lmos consta t speed, which was the same for all experiments. At he fixed end of the system the force was measured by tensile force pick-up (Philips PR 9226/02 max. load 2000 N) nd the displacement of the system, where the terior cruciat ligament was the only deformable structure was measured with tral ga ges (Philips PR 9810 tw coupled half bridge) mounted on flexible steel blade that was ben by pin on the plunger of the hydraulic system. The pick-up and the strain gauges were coupled t direct reading measuring bridges (Philips PT 1200) The signals from th two bridges were recorded by ink jet recorder (Siemens Oscillomink wi h load nd deformation on the ordinate of separate channels, the bscissa being time xes, nd by oscilloscope photograph (Tectronix 502 X Y-coupled, by Rollecord camera in a Ph lips adapter PM 9300 on orthochromatic glass plates, 6 9 cm Kodak O 800 or Ilford Selochrome) The measuring error as calcula ed from multiple pplications of know loads, for the whole measuring system was found t be 0.35 per cent calculated as the coefficient of variation. The corresponding figure for deformation measurements was 0.56 per cent. For both systems the measured signals were linearly proportional to load and deformation lues respectively

For the elasticity tests the clamps were fastened in pparatus (Fig 2) designed prevent ba klash and permit ery mall and accura deformation changes. Its mobile end consis ed of double-sided axial nd radial bearing fastened to shaft, one end of which was threaded and placed

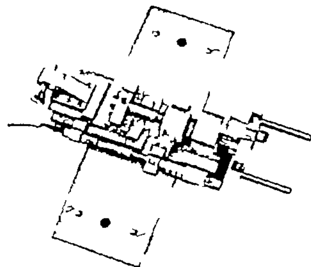
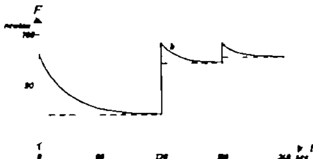


Fig 2. The backlash-f screw arrangement for the elastici tests. The specimen is fastened he left end of the pparatus The beef the righ and he d fferential tr nsformer ogether w th microm- meter on which is mou ore fastened re seen ow rds he bot- om of the picture

Fig. 3. Schematic diagram of the load relaxation phenomenon when load of 98.1 N has been applied initially. The load tends to fall towards an asymptote (the dotted line). After 120 sec the load is readjusted to 98.1 N and new relaxation phenomenon occurs, which is repeated after 60 sec. After another 60 sec the system is deloaded.



Inside fixed nut, which was, to prevent backlash sliced into three radial sections, each of which was pressed against the thread of the shaft by an external nut. The motion was performed by manual rotation of wheel at the end of the shaft, and as the pitch of the thread was 1.5 mm, the deformation changed this much each rotation of the wheel which was graduated to permit readings of 0.025 mm. The motion was recorded by displacement transducer of differential transformer type (Bofors RLK 1-5) and the corresponding load was measured at the fixed end of the experiment device by force transducer (Bofors KRK 1 max. load 200 N). Both transducers were coupled to direct reading measuring bridges and recordings were made as in the tensile strength experiments. The measuring error was found to be 0.33 per cent for the load and 0.42 per cent for the deformation measurements, when recording on the Oscillomink and 0.66 (load) and 0.28 (deformation) per cent respectively when recording from the oscilloscope. The elastic stiffness for this set-up was calculated to be 392 N/mm, including the spring constant of 981 N/mm of the force transducer and corrections were made in the elasticity parameters given.

In the elasticity tests the preparation was elongated until the load of 98.1 N was reached. Schematic load-time and elongation-time diagrams are given in Fig. 3 and 4. This value was selected as it is about one third of failure load and the load used by Viidik (1966). The deformation then achieved was kept constant for 120 sec during which time the load-relaxation phenomenon was registered. This period was sufficient to enable the calculation of the asymptote towards which the load tended to fall. Then the specimen was elongated until the load of 98.1 N was reached. Then after keeping the elongation reached, constant for another 60 sec the load was brought up to 98.1 N again by adjusting the elongation. After another sixty seconds the preparation was brought back to its original length. This is shown schematically in fig. 3—4. With 10 min resting interval — time deemed sufficient to recover the elastic after-effect (Viidik 1966) this whole three-step procedure was repeated twice, after which further changes with more cycles could not be anticipated (Viidik 1966). During the whole testing period the ligament was wrapped in saline-moistened gauze to prevent drying.

Mathematical and statistical method

From the tensile strength experiments the following parameters were analyzed: (1) tan — the coefficient of inclination for the linear portion of the curve, corresponding to the elastic stiffness of the material. (2) W — failure energy represented by the area between the load-deformation curve and the deformation axis. (3) P_{max}, the maximum load. (4) Δl_{max}, the deformation (an elongation at maximum load, calculated from the elongation at 50 N load level. (5) P_{lim}, the load value at which



Fig. 4. Schematic diagram for the elongation-time relationship for the load-relaxation phenomenon studied in fig. 3.

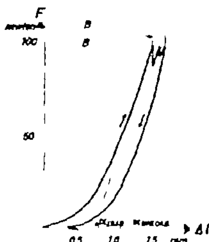


Fig. 5 Schematic diagram of an oscilloscope photograph of the loading and unloading phases of an experiment cycl The deformation distances measured and the angles estimated are indicated.

the linear portion of th load-deformation curve ends nd the curve levels off towards the deformation axis.

The gross shape of the curves was studied by cursory inspection of th oscilloscope photographs. Th tan and failure energy lues were calculated by computer The recordings from the Oscillomink were read for every millimeter change in the load curve and the corresponding defor mation values were read to the neares quarter of millimeter These data together with calibration data were processed by the computer First the mean tan alue for whole group was estimated assuming tha the linear parts of he load-deformation curves should have common starting point. Then the indi idual alues were estimated by the method of least squares. The standard error of the mean tan was estimated f om all indi idual measuring points on the load-deformation rves When calculating the failure energy values the load deformation curve was pproximated be linear between the points of measurement.

properties of the loading nd unloading phases of the experiments on elasticity were evaluated f om the oscilloscope photographs that displayed the load deformation rela ionships The following parameters were nalyzed. (f Fig 5 1) tan α, the coefficient of inclination, of the linear portions of the loading nd unloading curves, (2 B_1 th deformation to reach the 98 1 N load initially This and the following distances were measured from the poi of deformation t 1 N load as he exact zero point is ery difficul to est ma accurately (3 B_2 he deformation after the second load adjustment in loading cycle 4 B_3 B the distance covered in the tw load adjus ments 98 1 N.

The load-relaxation phenomena were evalu ed from the Oscillomink recordings. These ere read definite tervals of ime and he curve was read the nearest quarter of mm These data ere processed in computer A mathematical model was used to estimate the relevan fa s in he phenomena

$F(t) = F \exp(-\beta t) - F_a[1-\exp(-\beta t)]$

where

F = the initial load,

F = the asymptotic alue of F when → ∞

β = the shape parameter indicating the speed which the asymptot is reached

After estimating F_a, β was calculated w th the method of least squares. The correlation efficient of actual data to the thus evaluated equation was also calcula ed Differences bet een means of estimated parameters were ested by using Studen -test nd differences were regarded as significa t if $2P < 0.05$ and almost significan if $2P$ 0.10 o-sided tests

Results

Tensile stren, th

The gross shape of the load-deformation curves displayed an nitial toe part after which a fairly linear portion followed Some pecimens failed while in thi part of the curve and the curves of other levelled off somewhat towards the deformation axis

TABLE I Tensile strength parameters (mean values ± standard errors) for tibia — lig cruciatum anterius — femur preparations from trained and control rabbits. 2P < 0.10, 2P < 0.05. N = newton is the force unit in the International System of Units and 1 N = 0.102 kiloponds = 7.23 poundals

Parameter	Denomination	Trained	Controls
	—	23	21
tan	N/mm	214.0 ± 8.5	202.4 ± 9.4
W	Nmm	445.1 ± 48.9*	314.2 ± 33.7
P_{max}	N	317.2 ± 16.8	274.6 ± 17.5
ΔP_{max}	mm	1.7 ± 0.1	1.3 ± 0.1
P_{lin}	N	240.2 ± 14.7	221.2 ± 15.6

before failure occurred The failure occurred either abruptly or in 2 or 3 successive steps that followed each other rapidly No differences in the mode of failure could be seen between the trained and control groups.

The results from the analyses of the various parameters are given in Table I tanα showed a slight tendency to increase in the trained groups. The failure energy increased significantly (2P<0.05) and the maximum load and elongation at maximum load values tended to increase (2P<0.10) The maximum linear load values were indecisive the absolute values in N showed a slight tendency to increase but the values expressed in per cent of maximum load on the other hand showed a slight inclination to decrease.

The failure site on the specimens was mostly a bony attachment. In the control group 13 specimens failed as tear-off fractures of the tibia, four at the margin of the insertion of the ligament into the tibia with only very small bone fragments, two in the ligament and the remaining two as tear-off fractures of the femur In the trained group seventeen failed as tibial tear-off fractures, two at the margin of the tibial insertion two as femoral tear-off fracture and two in the ligament itself

Elasticity

The curves from the elasticity tests displayed the toe part towards the deformation axis in the beginning after which a linear portion ensued *cf* Fig 5 The *toe* part was more accentuated in the unloading curves, the linear portion was steeper the bending sharper and the toe part reached zero load when there was still some deformation left. No differences in the shape could be found between the trained and control groups.

The tan α values of loading and unloading are given in Table II No significant differences are to be found between the two groups although the values tend to be somewhat higher in the trained group. The tan α values for the loading phases increase significantly from the first to the second cycle while the unloading phases are unaffected The difference between the loading and unloading decreases correspondingly from the first to the second and third loading cycle

TABLE II The elastic stiffness (tan) for tibia — lig. cruciatum nterus — femur preparations when loaded to 98. 1 N and unloaded. Between each cycle there is 10 min resting time

	part	Trained	Controls
n		13	11
Cycle 1	loading	181.2 ± 4.9	180.5 ± 6.7
	unloading	288.5 ± 9.1	274.3 ± 11.1
	diff.	107.3 ± 9.7	93.7 ± 8.8
	loading	213.7 ± 3.9	12.8 ± 7.0
	unloading	291.2 ± 10.6	275.4 ± 12.5
	diff	77.5 ± 7.4	6..6 ± 6.1
3	loading	224.0 ± 6.4	217.4 ± 9.9
	unloading	285.5 ± 7.4	274.3 ± 10.2
	diff	61.5 ± 3.2	56.9 ± 4

The elongation parameters B_1 B_2 and B_2—B_1) are uninfluenced by training In both groups B_1 increases from cycle 1 to cycles 2 and 3 more than B_2 and correspondingly the difference B_2—B_1 diminishes. (Table III)

In the analysis of the load-relaxation phenomenon (Table IV) the asymptotic level toward which the load sinks was lower in the trained group in the first parts of all three testing cycles in Table IV a cycle 1 a 2P<0.10 2a 2P<0.0 and 3a <0.10 In the remaining parts of the testing cycles the tendency was also towards ower a mptotes in the trained group

The hape parameter tended to be higher in cycle 2a in the trained group than in the ontrol 2P <0.10 and to a lesser degree in cycle 1a. In the remaining cy le parts the alteratio.. were indecisive f Table IV b

Analy is a also made of the orrelation of the data calculated by th mputer with those ead from the experiment The correlation was found to be ry good.

TABLE III Deformation alues in mm for the experiments in T ble II For expla tion see text and Fig 5

	distance	Trained	Controls
n		13	11
C cle 1	B_1	1.09 ± 0.05	1.13 0.06
	B	1.25 ± 0.05	1.[illegible] ± 0.09
	B_2—B_1	0.1 ± 0.01	0.14 0.0[illegible]
	B_1	1.21 ± 0.05	1. [illegible] ± 0.09
	B_2	1.78 0.05	1.31 ± 0.06
	B_2—B_1	0.07 ± 0.01	0.07 ± 0.01
3	B_1	1.23 0.05	1.26 ± 0.09
	B_2	1.29 ± 0.05	1.33 ± 0.09
	B_2—B_1	0.0[illegible] ± 0.01	0.0[illegible] ± 0.01

TABLE IV —b. Parameters on load-relaxation in ligamentum cruciatum anterius from an initial load of 98.1 N (Mean values ± standard errors). For explanation see text and Fig. 3. Between each cycle there is 10 min rest. *2P < 0.10 *2P < 0.05

Asymptote in per cent of initial load

	Trained	Controls
	14	11
Cycle 1a	82.7±1.2*	85.8±1.2
1b	95.5±0 6	96.0±0 7
1	97 4±0.5	97 4±0 7
..a	90 7±0 7	92.8±0.7
2b	97 9±0.3	98.1±0.3
..c	98.5±0.2	98.8±0 3
3a	92.1±0 7	93.5±0 4
3b	98.3±0.3	98.5±0.3
3c	98.6±0.2	99 0±0.2

b Shape parameter

	Trained	Controls
	14	11
Cycle 1	—2.39±0.17	– 2.96±0.33
1b	—3.75±0 42	—3 08±0.32
1	—3 83±0.46	—3.25±0 59
2a	—2 17±0 17*	—2 84±0 29
2b	—3.84±0 59	—3 64±0 65
2c	—2 71±0.34	—2.76±0.37
3a	—2 40±0 26	–2 54±0.22
3b	—2 33±0.45	—2 56±0 38
3c	—2.45±0.38	—1 94±0 49

the correlation coefficient usually was between —0 99 and —0 85 For a few experiments, where the load relaxation phenomenon had been erv small the correlation was not so good.

Discussion

Our knowledge on what happens when a collagenous tru t is bjected to increased functional demand is based mainl n morphologi al n estigations The present investigation concerns the tensile strength nd lastict f the anterior cruciat ligament and ts attachments to femur and ti n the rabb It is shown that the energy required to rupture a tibia-lig cru ia m ant – f ur preparation (W_f) is significantl ncreased with training and the prepa at on aximum load nd deformation at maximum load alues tend to increase tl g These find ngs

are in agreement with those of Schild *et al.* (1967) who found the separation force for tibia—lig. collaterale mediale—femur preparations increase in rats when subjected to training. On the other hand the elastic stiffness (tan α) tends only slightly to increase. These findings can be accounted for by strengthening of the tibial and femoral epiphyses as the failure in almost all specimens occurred in the bony attachments and consequently before the maximum load of the ligament was reached. But the results from the elasticity tests imply that some change has happened in the ligamentous tissue too. The load-relaxation phenomenon is larger in the trained group. The asymptote (F) towards which the load sinks is lower in the trained group and the speed in this process represented by the shape parameter is higher. The deformation at which this phenomenon is studied is the same in both groups. These differences must be accounted for by changes in the ligamentous tissue the mechanical properties of which contain elastic viscous and plastic components (Viidik 1966). From these data it is so fa impossible to conclude how much the various components are altered quantitatively. To do this more information must be available on the interactions of the different components.

This investigation confirms also the results of previous reports that the bony attachment is the weakest point in a bone-ligament-bone preparation (Smith 1954, Viidik *et al.* 1965, Tipton *et al.* 1967) and shows that the site of this point is not altered by training although strengthening of it occurs.

mputer analysis programmed and statistical analysis supervised by E. Carlström, Ph. D

References

ANNOVAZZI, G. Osservazioni sulla elasticità dei legamenti. *Arch. Sci. biol.* (Napoli) 1928 *11* 467—502.

GRATZ, C. M., Tensile strength and elasticity tests on human fascia lata. *J. Bone Jt Surg.* 1931 *13* 334—340.

INGELMARK, B. E. Über den Ba der Sehnen während verschiedener Al ersperioden und unter verschiedenen funktionellen Bedingungen. *Upsala Läk.-Fören. Förh.* 1945. *50* 357—396.

INGELMARK, B. E. Der Ba der Sehnen während verschiedener Altersperioden und un er verschiedenen funktionellen Bedingungen. I. Eine quantitativ morphologische Untersuchung den Achillessehnen weisser Ratten. *Acta anat.* Basel 1948 113—140.

RIGBY, B. J., N. HIRAI, J. D. SPIKES and H. EYRING. The mechanical properties of ra tai tendon. *J. gen. Physiol.* 1959 60 *43* 265—283.

SCHILD, R. J., C. M. TIPTON nd R. J. TOM AK, Personal communication from Tipt 1967.

SMITH, J. W. The elastic properties of the an erior crucia ligament of the rabbit. *J. A* Lond 1954 *88* 369—380.

STUCKE, K. Über das elastische Verhalten der Achillessehne im Belastungsversuch. *Langenbeck Arch. klin. Chir.* 1950. 265 5 9—599.

TIPTON, C. M., R. S. SCHILD and A. E. F TT. Measurement of ligamentous strength in knees. *J. Bone Jt Surg.* 196 *49*-A 63— 2.

VIIDIK, A., Biomechanics and functional daption of tendons and join ligaments. In F. G. E ans (ed.) *Studies on the anatomy and function of bone and joints.* Springer. Berlin. 1966 pp. 17—39.

VIIDIK, A., Tensile strength properties of Achilles endon systems in trained and untrained rabbits. *Acta orthop. scand.* 1968 In press.

VIIDIK, A., The effect of training on th ensile strength of isola ed rabbit tendons. *Scand. J. plast. reconstr. Surg.* 1968 b. In press.

VIIDIK, A., L. SANDQVIST and M. MAGI. Influence of postmortal storage on tensile strength charac teristics and histology of rabbit ligaments. *Acta orthop. scand.* 1965 Suppl. 79 pp. 1—38.

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Rapid Uptake of Radiostrontium in Sperm

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Abstract

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$^{85}SrCl_2$ was administered to rams and semen collected. At 18—30 hrs after the i administration of the radionuclide there was maximal concentration in the spermatozoa. Already five hrs after the administration the spermatozoal content of ^{85}S was higher than the seminal content. From the higher content in the spermatozoa than the seminal plasma it was concluded that there might be considerable uptake of ^{85}Sr in the spermatozoa after the ejaculation. This was also shown to be the case when $^{85}SrCl_2$ was added to fresh sperm *in vitro*.

Previous investigations by Åberg and Gillner (1966 1967) deal w th radiostrontium of ram spermatozoa in relation to sperm DNA. In these papers attention was chiefly paid to the radiostrontium, which was incorporated during spermiogenesis, and which appeared in the semen about 40 days after th administration. Measurements starting 1 to 5 days after the administration showed a decreasing uptak of th nuclide in the ejaculated spermatozoa followed by slight increase on th 35—40th day The present investigation deals w th the relation of radiostrontium in the seminal plasma and the spermatozoa immediately after administration of $^{85}SrCl_2$ to rams. Furthermore the uptake of ^{85}Sr *in vitro* by ram spermatozoa was st died.

Three normal rams were njected i. each w th 1 mC ^{85}Sr. Semen was ollected with an artificial vagina and the radioactiv ty was measured n whol semen nd seminal plasma. The amount of ^{85}Sr in th spermatozoa was calculated from these figures and the sperm concentration Epididymal cont ts was collected accord ng to Crabo (1965) 2 nd 5 hrs aft the administration of ^{85}S. In order to study th uptake of ^{85}Sr in vitro ram semen was d l ed 1 4 w th sod m-potassium-phosphate buffer pH 7.2 conta ning f u se Dott nd Wh 1964 and $^{85}SrCl_2$ n an amount giving oughly 50 000 pm nl Inc bation w perf rmed t 37 C The sperm itality was judged with est mat motil nd dead li sperm counting (Hancock 1951) Some of the pr mples w old- h cked few minutes in an ic -bath.

Uptake of S ro The results are summarized F g 1 nd T bl I The e is an obvious apid uptake n the spermatozoa w th maxi al nc ntration 18—30

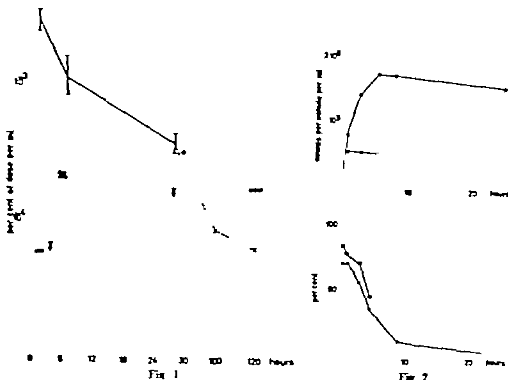


Fig. 1 *In vivo* uptake of ^{85}Sr in whole blood, semen and seminal plasma. The line represents the mean activity in whole blood (I = single determination), the symbols ○ □ ▽ the activity in whole semen and ● ■ ▼ the activity in seminal plasma from three rams.

Fig. 2 a. *In vitro* uptake of ^{85}Sr of ram sperm in Na-K-phosphate buffer at 37 C. ●—● = sperm, ○—○ = seminal plasma + buffer

Fig. 2 b. Motility and live-dead count of sperm. ●—● = per cent motility, □—□ = per cent unstained sperm.

hrs after the i. administration of the radionuclide. Five hrs after the administration the spermatozoa contain more ^{85}Sr than the seminal plasma. Investigations in the epididymal tail revealed a fairly high spermatozoal uptake with a sperm content which was 1.7 times higher than that of the epididymal plasma 5 hrs after the administration (Table I). As the ^{85}Sr content in ejaculated spermatozoa (Fig. 1) was maximally 1—10 times higher than the seminal plasma, it seems probable that a considerable uptake of ^{85}Sr to the spermatozoa takes place after the ejaculation. The higher figure was obtained with separation of sperm and plasma 40 min and the lower figure 10 min after the collection.

Uptake of ^{85}*Sr in vitro.* In fresh, untreated spermatozoa (Fig. 2) a considerable uptake took place reaching a maximal value 3—5 hrs after the dilution of the semen into the buffer. In cold-shocked spermatozoa (Fig. 3) there was also an uptake which was more rapid and declined more rapidly. It is known that mitochondria show a great affinity to strontium (Greenawalt and Carafoli 1966). A study on the

TABLE I. Concentration of ^{85}Sr (per cent of given dose $\times 10^{4}$ per ml) in packed spermatozoa and plasma from rams, and the ratio between the concentration in the spermatozoa and the plasma.

Hours after administration	Ejaculate			Epididymal tail		
	sperma-tozoa	plasma	spermatozoa / plasma	sperma-tozoa	plasma	spermatozoa / plasma
0— 4	0.7— 1 6	0.4—1.0	1.0— 2.5	0.4	0 4	1.0
4.5— 7	1 4— 2.1	0.7—1.9	1 1— 1 9	0.5	0.3	1 7
12— 30	1.3—13.6	1.3—2.0	0 9—10.0			
100—120	1 7— 2.9	1.3—1 6	1.9— 2.1			

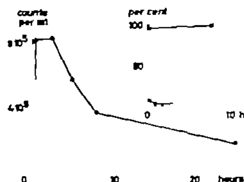


Fig. 3 a) *In vitro* uptake of ^{85}Sr of cold-shocked ram sperm in Na-K-phosphate buffer at 37 ●—●=sperm, ○—○=seminalplasma + buffer

Fig. 3 b) Motility and live-dead count of sperms. ●—●=sperm, □—□= per cent stained sperm.

relevance of mitochondrial accumulation of strontium for the uptake of this ion in intact sperm will be published elsewhere (H. Löw and B. Alm, in preparation) From the experiments here it can be concluded that the uptake of radiostrontium in the spermatozoa takes place both in the epididymis and in the semen. There is also an uptake in the testicular sperm cells, but it does not appear in the semen until later (Åberg and Gillner 1966) The immediate uptake is much higher than the testicular uptake and should be of greater importance in causing effects on the fertilized ovum if the uptake is localized to the mitochondria as the mitochondrial sheet in the middle piece of the sperm is incorporated in the ovum at fertilization (*cf* Blandau 1961)

References

ÅBERG, B and M GILLNER, Radiostrontium, radiophosphorus, stable strontium, stable calcium, DNA and DNA P in ram spermatozoa *Acta physiol scand* 1966 66 106—114

ÅBERG, B and M GILLNER, Radiostrontium, stable strontium, stable calcium and phosphorus in sperm DNA in Lenihan, Loutit & Martin (Ed) *Strontium metabolism* Academic Press Inc London. 1967

BLANDAU R. J Biology of eggs and implantation in Young, W C (Ed) *Sex and internal secretions*, Williams & Wilkins, N Y 1961 II, 707—807

CRABO B Studies on the composition of epididymal content in bulls and boars *Acta vet scand* 1965 6 Suppl. 5

DOTT H M and I G WHITE, Effect of potassium on ram spermatozoa studied by flow dialysis technique *J Reprod Fertil.* 1964 7 127—138

GREENAWALT J W and E CARAFOLI, Electron microscopy on the active accumulation of Sr by rat liver mitochondria *J Cell Biol.* 1966. 29 37—45.

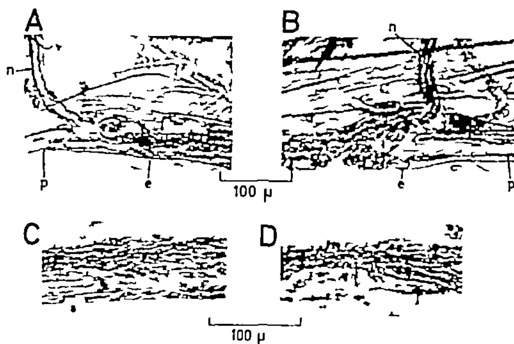


Fig. 1 Isolated muscle spindle with two (A) and with three (B) intrafusal muscle fibres. equatorial region, p. onset of the polar region, afferent erve.
Transition between polar and equatorial region i an intact muscle spindle (C) and after crushing the intrafusal muscle bundle in the equatorial region (D)

Stimulation The intrafusal muscle bundle was stimulated through glass capillary microelectrode (filled with 3M NaCl solution) placed 1—2 mm from the spindle on one of the nerve branches which supplied this spindle. The other electrode was an Ag AgCl wire of about 50 μ diameter placed in the Ringer bath. Rectangular pulses of 1—2 msec were applied as single stimuli and at rates of 25—30 sec

Results

Static length-tension diagram of isolated muscle sp ndles

Successful experiments were performed on ten muscle spindles, three 60—70 μ in diameter in the equatorial region and seven 75—107 μ in diameter (Fig 1 A, B) Although the number of intrafusal muscle fibres could not be determined with certainty in vivo the diameter as well as the microscopical appearance indicated that the 60—70 μ spindles contained two intrafusal muscle fibres and the larger spindles three intrafusal muscle fibres (Jahn 1959) The two-fibre spindles *lengthened 1.5—2 times more with load than the three-fibre spindles* The site of this difference was the equatorial region which was 2—4 times more compliant than the polar region (Fig 2) At high degrees of tretch the stiffness of the two and three fibre spindles was the same throughout The change in length as a f nction of load was the same in the two polar regions.

When the load exceeded 50 mg the change in length in the equatorial region was

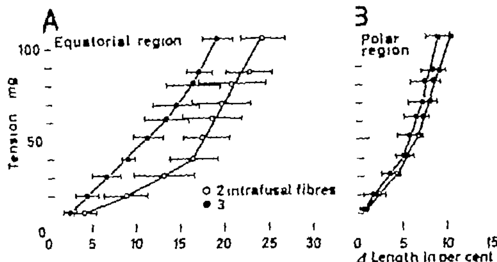


Fig. 2. Static length-tension diagrams of the equatorial region (A) and the polar regions (B) of 9 muscle spindles with 2 intrafusal muscle fibres and 7 with 3 intrafusal muscle fibres. The horizontal bars represent the standard error of the mean.

the same whether the load was stepwise increased or decreased (range 50—103 mg). When the load was less than 50 mg (i.e. at elongations below 10 per cent) the structural adjustment was slow and at a given length the tension obtained with release was less than with stretch. After removal of the load an elongation of 6—8 per cent persisted (Fig. 3 A). In the polar regions the length-tension diagram was the same whether the spindle was stretched or released.

One to one and a half hours after the muscle spindle was damaged by squeezing the equatorial region between a wedge of 50—80 and a perspex plate the compliance of the equatorial and polar regions was two to three times greater than in the intact spindle. The static length-tension diagram was then the same in "two-fibre" as in the "three-fibre" spindles (Fig. 3 B and C). In the polar region close to the site of damage either the cross striation of the intrafusal muscle fibres had disappeared or the fibres had retracted. The intrafusal nerve branches were still expanded over the perifascicular space indicating that they were attached to the intrafusal connective tissue (Fig. 1 D).

Intact muscle spindles did not break at the greatest load available (485 mg). Three damaged spindles ruptured at the site of injury when the load was 120—140 mg and the equatorial region was stretched 35—40 per cent above equilibrium length.

Static modulus of elasticity (E)

To compare the elastic properties of muscle spindles and of extrafusal muscle fibres the slope of the length-tension diagram per unit cross-sectional area was determined

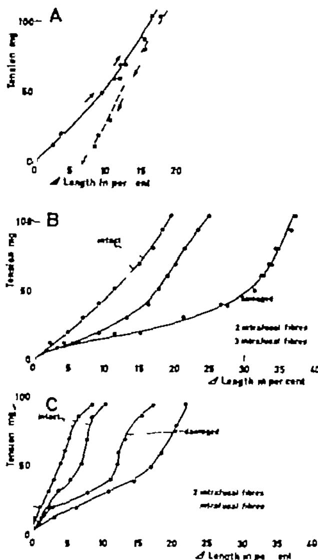


Fig 3 A Length-tension diagram of muscle spindle with 3 intrafusal muscle fibres. The arrows indicate an increase and decrease in tension at a rate of 10 mg/min (17—20 C)
B Static length-tension diagram of the equatorial region of two muscle spindles before and 1 1/2 hrs after crushing of the intrafusal muscle fibres. The diameter in the equatorial region of the intact two-fibre spindle was 70 μ and of the three-fibre spindle 100 μ.
C Static length-tension diagrams of the polar region of two muscle spindles before and 1 1/2 hrs after crushing of the intrafusal muscle fibres. The diameter in the polar region of the two-fibre spindle was 28 μ and of the three-fibre spindle 43 μ.

in the equatorial and polar regions. These slopes are denoted as modulus of elasticity" (E) In seven muscle spindles with three intrafusal muscle fibres E was 9 times less in the equatorial than in the polar regions. In three spindles with two intrafusal muscle fibres the difference was still greater (Fig. 4) In the 10 muscle spindles examined E averaged in the equatorial region $5.9 \pm 1.1 \times 10^4$ dynes/cm² (18—21 C) at an elongation of 10 per cent above equilibrium length. This is about 10 per cent above eq ilibri m length This is bout 10 times greater than the elasticity modulus of isolated extrafusal muscle fibres of the frog (Buchthal 1942, Buchthal, Kaiser and Rosenfalck 1951) and of whole muscle measured at equilibrium length (Walter 1944—1947) In the polar region stretched 10 per cent E

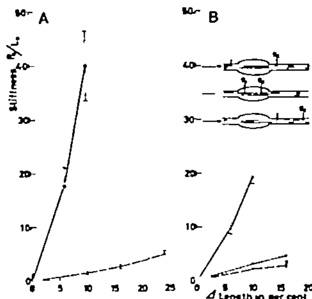


Fig. 4. Static stiffness of the equatorial and of the polar region as function of length, A in spindles with two and B with three intrafusal muscle fibres. The scheme to the right indicates the region to which the diagram refers.

averaged 7.6 ± 1.1 × 10 dynes/cm². This modulus of elasticity is still 100 times less than that of the tendon (Wöhlisch, du Mesnil de Rochemont and Gerschler 1926).

Twitch and tetanus of intrafusal muscle fibres

The spontaneous contractions of single intrafusal muscle fibres with a diameter of 13—14 μ lasted about 200 msec (5 spindles 17—20 C). The peak was reached after 40—45 msec (Fig. 5 A, Table I). The twitch tension varied in different muscle spindles between 0.7 and 1.3 mg and in the single muscle spindle by ± 15 per cent. The time from the peak to half-relaxation of the twitch lasted 40—55 msec (17—20 C). The time course of the movement artefact accompanying intrafusal recording of action potentials from single or two intrafusal muscle fibres was similar to that of the twitch tension (Fig. 5 B).

In half the spontaneous contractions the tension rose more slowly (rising phase 60—70 msec) and was lower (0.2—0.4 mg, Table I). These contractions were probably non-propagated responses (Jahn 1968).

The tension of the intrafusal muscle bundle evoked by stimulus to the motor nerve rose within 50—60 msec to a peak of 2—4 mg. The tetanus tension was 11—15 mg (Fig. 5 IV). The twitch tetanus ratio was 1 : 5 in one two-fibre spindle and 1 : 3 to 1 : 4 in three three-fibre spindles. With a fibre diameter of 13.5 μ (Jahn 1959) and 2 or 3 intrafusal muscle fibres per muscle bundle the maximum tetanic tension (P) was 3.4—3.9 kg/cm. The rate of tension development was 40—70 mg/sec, i.e. about 4 P₀/sec.

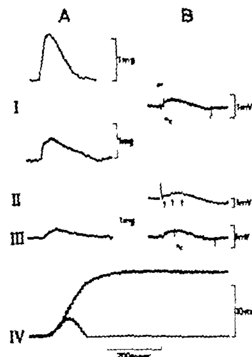


Fig. 5 A Mechanical responses associated with spontaneous contractions of single intrafusal muscle fibres the contractions in *I* were probably propagated, in III local (17—20 °C)
B Movement artefacts during intrafusal recording of action potentials from the muscle spindle. *I* and *III* spontaneous contraction of single intrafusal muscle fibres, II at least two intrafusal muscle fibres.
m action potentials from intrafusal muscle fibres, a_0 and mw evoked afferent action potentials (cf. Jahn 1968 a)
IV Twitch and tetanic tension of a bundle with 3 intrafusal muscle fibres evoked by stimuli to the motor nerve.

Table 1 Tension development in extrafusal and in intrafusal twitch fibres

twitch tension mg	diameter of muscle fibre µ	time to peak msec	time to half relaxation msec	max. tetanic tension kg/cm	temp °C	reference
	intrafusal fibres in isolated muscle spindles					
a) 0.7—1.3	13—14	40—45	40—55	3.4—3.9	17—20	FROG
b) 0.2—0.4	13—14	60—70	70—80			this study
0.05—0.5	4—15	180—250	300—500	(0.6—3.6)	20	CAT Diete Spiff 1967
	isolated extrafusal muscle fibres					
117—153	93—126	35—40	ca 45		18—22	FROG Håkansson 1957
	65—100			3.5	20	FROG Casella 1935
18—115	[illegible]0—110	29—54	34—39	3.1—3.3	21—23	TOAD (XENOPUS) Lannergren and Smith 1966

a) probably propagated contraction
b) probably local contraction

Discussion

The complex morphological structure of the muscle spindle makes it difficult to predict whether its compliance is the same throughout or differs at the site of the sensory ending. The measurements of this study have shown that the polar region at the periphery of the encapsulated zone is less compliant than the equatorial region though its diameter is smaller. When the intrafusal muscle fibres in the equatorial region were crushed the stiffness of the muscle spindle was diminished to less than half both in the equatorial and in the polar region because the muscle fibre content and the sarcolemma no longer contributed to the compliance. The differences in compliance along the length of the intact muscle spindle are consistent with the two zones of different structure in the intrafusal muscle fibres, the compact and the reticular zones (Katz 1961). In addition there are many elastic fibres along the muscle spindle in the innervation areas of frog spindles (Karlsson, Andersson-Cedergren and Ottoson 1966). In mammalian muscle spindles elastic fibres are sparse in the centre of the equatorial region around the nuclear bag and profuse at the spindle poles (Cooper and Daniel 1967). Crustacean stretch receptors have a layer of connective tissue ground substance intimately apposed to the sarcolemma of the muscle fibres (Bodian and Bergman 1962). The maximum load applied to the receptor muscle of crayfish stretch receptors was 10 mg (Krnjević and van Gelder 1961) and 50 mg (Wendler and Burkhardt 1961). The elastic modulus of crustacean stretch receptors is 0.7–7.0×10^6 dynes/cm^2 20–30 per cent above zero length (Krnjević and van Gelder 1961), the highest value being of the same order as in the equatorial region of the frog muscle spindle stretched 10 per cent above equilibrium length. My finding of a greater stiffness of the muscle spindle than of extrafusal fibres and the finding of a nearly identical compliance for the spindle capsule and the intrafusal muscle fibres are in agreement with the indirect evidence obtained from cross-sections of cat muscles fixed while subjected to acute stretch. Nuclear chain and bag fibres as well as the capsules participated almost equally in lengthening, but none of these structures was stretched as much as the extrafusal tissue (Bridgeman, Sweeney and Eldred 1966). A higher stiffness of the polar than of the equatorial region of the spindle might account for the higher threshold of the secondary than of the primary endings in mammalian muscle (Bessou and Laporte 1962, Matthews 1962). That stimulation of some fusimotor fibres causes an increase in the spontaneous afferent activity and stimulation of others causes an enhanced sensitivity to stretch was ascribed to the type of contraction evoked (Bessou and Laporte 1966, Schafer and Henatsch 1967). A localized contraction probably does not affect the spontaneous afferent activity because the length of the region of the primary sensory ending remains practically unchanged. A local contraction may however increase the sensitivity to stretch. The greater stiffness of the contracted regions causes a larger part of the external stretch to act on the sensory ending. A contraction comprising most of the intrafusal fibre stretches the region of the sensory endings and results in an increase in spontaneous aff

activity. At the same time the sensitivity to stretch is reduced because the increase in stiffness involves almost the entire length of the spindle. The differences in elastic properties found in different parts of the muscle spindle support these explanations.

The higher "modulus of elasticity" of the different regions of the muscle spindle as compared with the modulus of extrafusal muscle fibres (p. 5) might either be due to the presence of parallel elastic material such as the capsule and the longitudinal elastic fibres or to differences in the two types of muscle fibres (extrafusal and intrafusal). To test the latter assumption would require isolation of intrafusal muscle fibres throughout the encapsulated portion of the spindle.

As estimated from the twitch tension and the static length tension diagram, the deformation of the sensory region caused by the spontaneous contraction of an intrafusal muscle fibre is 1 per cent. Assuming the elasticity of the capsule to remain constant and the sensory region to extend over about 300 μ (Jahn 1959) a stretch of 1 per cent would correspond to an elongation of 3 μ. Correcting for the dynamic conditions during a twitch by assuming the dynamic stiffness to be three times greater than the static (Buchthal *et al.* 1951) the deformation of the sensory region caused by a spontaneous intrafusal contraction is 0.3 per cent, corresponding to about 1 μ. Since the contraction of a single intrafusal muscle fibre regularly evoked an afferent discharge (Jahn 1968 a) this deformation of the sensory region gives a rough estimate of the threshold of the muscle spindle. This is of the same order as the threshold calculated from measurements of the minimum deformation which ...sed an afferent response to every transient stretch applied at a repeat rate below ...per second (Jahn 1966 and 1968 b).

The time to peak and to half relaxation of the twitch of spontaneously contracting intrafusal muscle fibres was about the same as in extrafusal twitch fibres of frog and toad (for ref. see Table I). The twitch tension of intrafusal muscle fibres of the cat is of the same order as in the frog (Diete-Spiff 1967). The smaller peak tension of intrafusal than of extrafusal twitch fibres can be accounted for by the smaller diameter of the intrafusal muscle fibres, the isometric tension per unit area being the same as in extrafusal fibres (for ref. see Table I).

In *Xenopus* the twitch tension of an intrafusal muscle bundle was 10—25 mg (20—23° C; Smith 1964 b). This tension seems to be high because the bundle contains on the average three intrafusal muscle fibres with similar diameters as in frog spindles (Jahn 1968 a). Moreover when the diameters were the same the peak tensions of extrafusal twitch fibres were of the same order in *Xenopus* and in the frog (Table I). Therefore the high tension observed by Smith (1964 b) in the intrafusal bundle of *Xenopus* was probably due to interference of remaining extrafusal tissue. The twitch tension of the intrafusal bundle of a frog muscle spindle (2—4 mg) was lower than that of the fast muscle bundle of lobster stretch receptors (1?—13 mg); the time to peak (20—30 msec) was shorter than in the frog (Kuffler 1954). These measurements were carried out at a higher temperature (25° C) and according to morphological pictures of crayfish stretch receptors (Bodian and Bergman 1962) the number of muscle fibres in the fast bundle is about 12.

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References

Bessou P and Y Laporte, *Observations on static fusimot fibres. Muscular Afferents nd Motor Control* pp. 81—89 (ed. Granit, R., Almqvist and Wiksell, Stockholm 1966)
Bridgm C, S Sweeney and E. Eldred, The effects of contraction and stretch of muscle on morphology of its spindles. *A at. Rec* 1966 *156* 67—82.
Buchthal, F The mechanical properties of the single striated muscle fibre at rest and during contraction and their structural interpretation. *Dan. Biol. Medd* 1942. *17* no. 2. 140 pp.
Buchthal, F E. Kaiser and G. G Knappeis Elasticity viscosity and plasticity in the cross striated muscle fibre. *Acta physiol. scand* 1944 *8* 16—37
Buchthal, F E. Kaiser and P Rosenfalck, The rheology of the cross striated muscle fibre with particular refrence to isotonic conditions. *Dan Biol. Medd* 1951 *21* 318 pp.
Casella, C., Tensile force in total striated muscle isolated fibre and sarcolemma. *Acta physiol. scand* 1951 *21* 380—401
Cooper, S. and P M. Daniel, Elastic tissue in muscle spindles of man and rat. *J Physiol.* (Lond.) 1967 *192* 10 P
Diete-Spiff K Tension development by isolated muscle spindles of the cat. *J Physiol* (Lond.) 1967 *193* 31—43.
Håkansson, C. H., Action potential and mechanical response of isolated cross striated frog muscle fibres at different degrees of stretch. *Act physiol. scand* 1957 *41* 1—18.
Houk, J C., R. W Cornew and L. Stark, A model of adaptation in amphibian spindle receptors. *J theor Biol.* 1966. *1* 196—215.
Jahn U, Morphological observations on living neuromuscular spindles. *Act anat* (Basel) 1959 *39* 341—350.
Jahn, S. Al Azharia, Spontaneous intrafusal contractions and spontaneous afferent discharges as tool in the analysis of muscle spindles. *Muscular Afferents and Motor Cont ol* pp. 453—456 (ed. Granit, R., Almqvist and Wiksell Stockholm 1966)
Jahn, S. Al Azharia, Spontaneous afferent discharges and spontaneous intrafusal contractions in isolated muscle spindles of the frog. *Act physiol. sc d.* 1968 a. *72* 350—365
Jahn S Al Azharia, Response of isolated muscle spindles to single transient stretches. *Act physiol. scand.* 1968 b. *72* 441—455.
Jahn, S Al Azharia, The excitability of frog isolated muscle spindles during repetitive stretches. *Act physiol. sc d.* 1968 d. *73* 418—426.
Karlsson, U E Andersson-Cedergren and D Ottoson, Cellular organization of the frog muscle spindle as revealed by serial sections for electron microscopy *J Ultrastruct Res.* 1966. *14* 1—35
Katz, B The terminations of the afferent nerv fibre in the muscle spindle of the frog. *Phil. Trans. B.* 1961 *243* 221—240
Kuffler, S. W and C. Eyzaguirre... Ka jevih, K and H M. v Gelder, Tension changes in crayfish stretch receptors. *J Physiol.* (Lond.) 1961 *159* 310—325.
K ler, S. W Mechanisms of activation and motor control f stretch receptors in lobster and crayfish. *J Neurophysiol.* 1954 *17* 558—574
Lännergren, J and R. S. Smith, Types of muscle fibres in toad skeletal muscle. *A t physiol. scand* 1966 *68* 263—274
Matthews, P B C., The differentiation of two types of fusimotor fibres by their effects on the dynamic response of muscle spindle primary endings. *Quart J p Physiol* 1962 *47* 324—333
Schäfer, Sonja S and H. D Henatsch Dehnungsantworten der primaren Muskelspindel-Afferenz bei elektrischer Reizung und naturlicher Innervation der beiden fusimotorischen Fasertypen. *E p. Brain Res* 1968 *4* 275—291
Smith, R. S Contraction in intrafusal muscle fibres of Xenopus laevis following stimulation of their motor nerves. *Acta physiol sc d* 1964 b *62* 195—208.
Talbot S. A J L. Lilienthal, J Beser J and L W Reynolds, A wide range mechano-electric transducer for physiological pplications. *Rev Sci Instr* 1951 *22* 233—236
Wal er, W G The tensile strength of stria ed muscle investigated on the gastrocnemius muscle of the frog. *A h aderl d Physiol.* 1944—1947 *8* 655—669
W ndler, L and D Burkhardt Zeitlich abklingende Vorgange in der Wirkungskett zwischen Reiz und Erregung (Versuche an abdominalen Streckreceptoren dekapoder Krebse) *Z. Naturf* 1961 *16 b* 464—469
Wöhlisch E., R. du Mesnil de Rochemont nd H G ... Untersuchungen über die elastischen Eigenschaften tierischer Gewebe I *Z Biol.* 1926 *85* 325—341

Acta physiol. scand. 1968, 74 394—397

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Formation of Extracellular Adenosine Triphosphate by Yeast Cells

By

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Received 28 February 1968

Abstract

Ågren, G. and G Ronquist *Formation of ext acellular adenosine triphosphate by yeast cells* Acta physiol. scand. 1968, 74 394—397

Yeast cells were incubated with the necessary substrates and cofactors of the glyceraldehyde 3-phosphat dehydrogenase and phosphoglycerate kinase reactions (complete system) and also with an incomplete system lacking the phosphorylated substrates and cofactors. Ten times more ATP was formed by the yeast cells incubated with the complete system as compared w th those incubated with the incomplete system. All the resulting ATP formed by the phosphorylated substrates and cofactors present in the complete system could be recovered in he extracellular medium. Only an insignificant part of this ATP has probably been formed y enzymes of intrac llular origin. It is reasonable to assume hat most of the ATP present in the extracellular medium has been formed by the enzymes located on the surface of the yeast cells.

Work in this laboratory (Ronquist 1968) has shown that intact human erythrocytes are capable of forming extracellular ATP when incubated with all the substrates and cofactors of the glyceraldehyde 3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate NAD oxidoreductase (phosphorylating) E.C. 1.2.1 12) and phosphoglycerate kinase (ATP D-3-phosphoglycerat 1-phosphotransferase E.C. 2 7 2.3) reactions. It was therefore of interest to see if this property of human red cells was shared by other cells. This paper describes th formation of extracellular ATP by yeast cells.

Material and methods

Fresh baker east strain of Saccharomyces cerevisiae was suspended in 0 15 M sodium hloride (1 5 wet weight in g olume in ml and centrifuged for 5 min at 450 g This and all following preparatory steps were performed t 4 C. The supernatant ontaining cell debris and cells was discarded. The bottom cell-lay in he tube was resuspended in similar proportions of 0.15 M NaCl as abov and centrifuged for 10 min 650 × g The sedimented cells were finally washed once with distilled w ter before use

The washed and packed yeast cells were suspended in 10 olumes (wet weight (g)/volume (ml)) of 10^{-2} M Tris-acetic acid buffer pH 7 5. The ll suspension was brought to 37 C before incubation. In some experiments the cell suspension was preincuba ed for 15 min in

[1] Abbreviations: Tris = Tris(hydroxymethyl)aminomethane ^{32}P = ^{32}P -orthophosphate; UV = ultraviolet nMoles = nanoMoles (1 × 10 M les

TABLE I $^{32}P_i$ incorporation into ADP and ATP by yeast cells incubated in the complete system. Yeast cells, suspended in 10 volumes (wet weight (g)/volume (ml)) of 10^{-2} M Tris-acetic acid buffer pH 7.5 incubated for various times at 37 °C in the complete or incomplete systems (see text). Final volume of incubation medium was 20 ml. The incubations were terminated by adding 4.5 ml of 4 N perchloric acid to the media. The labelled nucleotides were separated and analysed as given in the text. The $^{32}P_i$ incorporation into ADP and ATP is expressed in *nanoMoles of orthophosphate per gram dry weight of yeast cells.*

	Incubation time (minutes)	AD^{32}P (nMoles per g)	AT^{32}P (nMoles per g)
Complete system	1	0.49	1.14
	10	1.0[illegible]	2.91
	20	1.52	4.64
Incomplete system	1	0.10	0.15
	10	0.16	0.27
	20	0.22	0.42

TABLE II The extra- and intracellular distribution of labelled nucleotides of yeast cells incubated in the complete and incomplete system. Yeast cells, suspended in 10 volumes (wet weight (g)/volume (ml)) of 10^{-2}M Tris-acetic acid buffer pH 7.5 were incubated for various times at 37 °C in the complete or incomplete systems (see text). The final incubation volume was 20 ml. At the end of the incubation periods the cell suspensions were centrifuged for 10 min at 650 × g. The clear supernatants (extracellular media) were withdrawn from the packed cell phases (intracellular media). The labelled nucleotides (ADP and ATP) were separated and analysed as given in the text. The $^{32}P_i$ incorporation into ADP and ATP is expressed in nanoMoles per gram dry weight of yeast cells.

	Extracellular medium		Intracellular medium	
	AD^{32}P (nMoles per g)	AT^{32}P (nMoles per g)	AD^{32}P (nMoles per g)	AT^{32}P (nMoles per g)
Complete system				
1 min	0.58	1.32	0.14	0.32
10 min	1.17	3.84	0.19	0.57
20 min	1.67	6.17	0.3[illegible]	0.91
Incomplete system				
1 min	0.00	0.02	0.10	0.21
10 min	0.01	0.02	0.14	0.39
20 min	0.01	0.07	0.19	0.48

oxygen before the anaerobic incubation with the substrates and cofactors of the glyceraldehyde-phosphate dehydrogenase and phosphoglycerate kinase reactions. All incubations were performed at 37 °C for various time intervals in nitrogen gas atmosphere. The incubation medium was the following

Tris-acetic acid buffer pH 7.5 300 µmoles, $MgCl_2$ 25 µmoles, glutathione (reduced form) 5 µmoles, glyceraldehyde-3-phosphoric acid 5.9 µmoles, ^{32}P 2 µmoles, NAD^+ 5.- µmoles, ADP 14 µmoles. An incubation medium containing all these substances is called the "complete system". Alternatively the yeast cell suspension was incubated with only the Tris buffer, $MgCl_2$, glutathione and ^{32}P. This is referred to as the "incomplete system". The final incubation volume was 20 ml. The ^{32}P was purified before use as described previously (Ronquist 1957). The incubations were carried out in polyethylene plastic tubes with gentle bubbling of nitrogen gas into the medium. At the end of the incubation periods some tubes, containing the yeast cells together with either the complete or incomplete system, were stopped directly by adding 4.5 ml of 4 N perchloric acid to the medium followed by 10 ml of ice-cold distilled water (see Table I). The precipitated yeast cells were centrifuged and filtered before neutralization with potassium hydroxide.

In the other experiments (see Table II) the yeast cells suspended in the complete and incomplete systems, respectively, were centrifuged for 10 min at 650 × g directly at the end of the incubation period. The supernatants ("extracellular mediums") were decanted into tubes containing 1.5 ml of 3 N perchloric acid and were then neutralized with potassium hydroxide. The remaining, packed yeast cells ("intracellular mediums") were immediately precipitated with 4.5 ml of 4 N perchloric acid. 10 ml of ice-cold water was added to the precipitated cells before filtration and neutralization with potassium hydroxide.

To determine any enzyme leakage out of the cells 20 ml fractions of the yeast cells suspended in the Tris buffer were centrifuged at 3 C without contact with the complete or incomplete system. The clear supernatants were incubated with the complete system under the same conditions as mentioned above. This was the "blank".

Labelled ADP and ATP were separated and identified as previously described (Ronquist 1957).

Results and discussion

Table I shows that there is a significant difference between the amounts of labelled ATP formed by the two systems. The observed effects could be due to "cell-surface reactions" if it is assumed that the phosphorylated substrates and cofactors do not penetrate the cell membrane.

It was therefore necessary to exclude any secretion or diffusion of intracellular enzymes to the surrounding medium. It was further of interest to see how the labelled products were distributed between the intra- and extracellular spaces.

Data given in Table II show that most or all of the AT^{32}P formed by yeast cells incubated in the complete system is distributed in the extracellular medium. The small amounts of labelled ATP formed in the intracellular medium might be explained by a very slow penetration of ^{32}P into the yeast cells. At 20 min incubation only 3 per cent of the total ^{32}P was recovered in the cell phase. In addition there was no clear difference in the ^{32}P distribution between the yeast cells incubated in the complete and incomplete systems. Thus it cannot be excluded that the ^{32}P of the cell phases might have been adsorbed on the cell surface. These findings are in agreement with earlier investigators (Hevesy, Linderstrøm-Lang and Nielsen 193[illegible], Goodman and Rothstein 1957, Swenson 1960, Schönherr and Borst-Pauwels 196[illegible]) who found no or very little penetration of ^{32}P through the yeast cell membrane.

Only small amounts of labelled ATP were formed by the blank (Table III). Therefore it is not probable that the AT^{32}P formed by the yeast cells incubated in the complete system is due to intracellular enzymes which have leaked out of the cells. It is reasonable to assume that the observed effects are the results of reactions on the cell surface.

TABLE III. Supernatant separated from the yeast cell suspension and incubated in the complete system. Yeast cells, suspended in 10 volumes (wet weight (g)/volume (ml)) of 10^{-2} M Tris-acetic acid buffer pH 7.5, centrifuged at 37° C for 10 min at 650 × g. The clear supernatant obtained (blank) was incubated for various times at 37° C with the complete system. Final incubation volume was 18 ml. The incubations were terminated by adding 3 ml of 3 N perchloric acid to the media. The $^{32}P_i$ incorporation into ADP and ATP is expressed in nanoMoles of orthophosphate per g dry weight of yeast cells.

	AD³²P (nMoles per g)	AT³²P (nMoles per g)
1 min	0.05	0.09
10 min	0.10	0.36
20 min	0.16	0.52

In some experiments the yeast cells were preincubated in oxygen for 15 min before the anaerobic incubation with the complete system. The interesting observation was made that after this pretreatment the amount of extracellular AT³²P formed by the cells decreased to about 50 per cent. This observation is in contrast with the finding of Rosenthal and Lasnitzki (1928). They showed that a short period of preincubation in oxygen is followed by a marked increase in the subsequent glycolysis under anaerobic conditions. However the present investigation deals with only two enzymatic steps of glycolysis. Further the surface-located enzymes might be more sensitive to the treatment with oxygen than the intracellular enzymes.

ADP was also labelled as shown in the Tables. It suggests the presence of adenylate kinase (ATP AMP phosphotransferase, E.C.2.7.4.3) on the yeast cell surface. It also agrees with the finding that more extracellular AT³²P was formed (according to UV and total phosphorus determination) by the yeast cells incubated in the complete system than could be calculated from the radiochemical data. Thus this additional extracellular ATP has probably been formed in the adenylate kinase reaction.

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References

Goodman, J. and A. Rothstein, The active transport of phosphate into the yeast cell. *J. gen. Physiol.* 1957. *40*. 915—923.

Hevesy, G., K. Linderstrøm Lang and N. Nielsen, Phosphorus exchange in yeast. *Nature (Lond.)* 1937. *140*. 725.

Ronquist, G., Formation of adenosine triphosphate by a membrane fraction from human erythrocytes. *Acta chem. scand.* 1967. *21*. 1484—1494.

Ronquist, G., Formation of extracellular adenosine triphosphate by human erythrocytes. *Acta physiol. scand.* 1968. *74*. In press.

Rosenthal, O. and A. Lasnitzki, Über den Stoffwechsel stationärer und wachsender Gewebe. *Biochem. Z.* 1928. *196*. 340—425.

Schönherr, O. Th. and G. W. F. H. Borst Pauwels, Investigation into the permeability of yeast cells to phosphate. *Biochim. Biophys. Acta* (Amst.) 1967. *135*. 787—790.

Swenson, P. A., Leakage of phosphate compounds from ultraviolet-irradiated yeast cells. *J. cell. comp. Physiol.* 1960. *56*. 77—91.

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The Lactate/Pyruvate Ratios of Cerebrospinal Fluid of Rats and Cats Related to the Lactate/Pyruvate, the ATP/ADP, and the Phosphocreatine/Creatine Ratios of Brain Tissue

By

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Abstract

Granholm, L., A. E. Kaasik, L. Nilsson and B. K. Siesjö *The lactate/pyruvate ratios of cerebrospinal fluid of rats and cats related to the lactate/pyruvate the ATP/ADP and the phosphocreatine/creatine ratios of brain tissue* Acta physiol. scand. 1968. 74 398—409

The CSF and brain tissue concentrations of lactate and pyruvate were measured in rats and rats and related to the corresponding tissue concentrations of ATP ADP phosphocreatine and creatine A comparison between superficial and deep phenobarbital anaesthesia, and nitrous oxide anaesthesia, showed that the type of anaesthesia had marked effect on the tissue metabolites with a differential influence on the lactate/pyruvate and on the ATP/ADP and the phosphocreatine/creatine ratios Optimal conditions for freezing and extracting the tissue were sought and were found to include freezing of the head in such way that the ventilation and the circulation of the animals were upheld during the freezing, as well as extraction of the tissue at −15 to −20 C. The distribution of lactate and pyruvate between extra- and intracellular phases was investigated in both rats and cats. In all groups studied lactate and pyruvate were found in higher concentrations in CSF than in the tissue suggesting a pH-dependent distribution of the weak acids In the cat, the CSF pyruvate occurred in relatively higher concentration than lactate indicating specific transport mechanism for pyruvic acid

Recent studies of the energy metabolism of tissues and tissue systems have clearly indicated that the overall oxidation/reduction states of cytoplasmatic and mitochondrial phases can be deduced from analyses of the appropriate redox systems (Huckabee 1958, Hohorst 1960 Williamson, Lund and Krebs 1967) Among these the lactate/pyruvate system has a special importance since its components are supposed to be freely diffusible Consequently analyses of lactate and pyruvate in the extracellular phases may be useful for studies of hypoxia in tissues. Thus, it has recently been shown that measures which are apt to produce brain hypoxia lead to

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increases in the cerebrospinal fluid (CSF) lactate/pyruvate ratio (Siesjö, Kjällquist and Zwetnow 1968). However, before such measurements can be adequately interpreted in terms of tissue redox changes the relations between the lactate/pyruvate systems in the CSF and in the tissue must be determined. Moreover, changes in the tissue lactate/pyruvate system must be related to other metabolic sequelae of tissue hypoxia such as those affecting the balance between synthesis and breakdown of ATP and phosphocreatine.

In the present experiments the CSF lactate/pyruvate ratio of cats and rats were related to the corresponding tissue ratios, and to the tissue concentrations of ATP, ADP, phosphocreatine and creatine. An analysis was also made of the relation between the lactate and pyruvate concentrations in the extra- and intracellular spaces, and the corresponding hydrogen ion gradients. The relation between the lactate and pyruvate concentrations in CSF and brain tissue does not seem to have been studied outside the present laboratory (see preliminary communication by Granholm and Siesjö 1967), neither can tissue concentrations for the other metabolites concerned be adequately deduced from previous reports. Thus, although specific enzymatic methods have become available for the determination of a number of intermediary tissue metabolites (Thorn *et al.* 1958, Hohorst, Kreutz and Bücher 1959, Lowry *et al.* 1964, Schmal *et al.* 1965), the results vary with the species studied, the anesthetic used, and the freezing method employed. In the present report both rats and cats were used. The cats were anesthetized with phenobarbital, while the rats were anesthetized either with nitrous oxide or with intraperitoneal phenobarbital. The tissues were frozen *in situ* with a technique that allowed the lung ventilation and the arterial blood pressure to be upheld until the whole brain was frozen. The results show that both the type and concentration of the anesthetic and the freezing method have marked effects on the metabolite concentrations measured.

Methods

The experiments were made on cats weighing 2—3.5 kg, and on male rats of the Wistar strain, weighing 300—400 g. The cats were anesthetized with phenobarbital (100 mg/kg i.p.), tracheotomized, and allowed to breathe room air spontaneously. One femoral artery was cannulated for blood sampling and for blood pressure recording with an electromanometer (Elema, Stockholm). The atlanto-occipital membrane was exposed for sampling of CSF. A craniotomy was made over the parietal lobes but the dura was generally left intact, and care was taken not to damage the brain surface. The brain was frozen by pouring liquid nitrogen into a plastic funnel, fitted into a skin incision over the craniotomy. With this freezing technique (Kerr 1935, Stone 1938) the lung ventilation and the heart activity were upheld for 10—20 min. The head of the animal was then usually frozen in liquid nitrogen for another 15—20 min, after which time the brain was chiselled out using instruments cooled in liquid nitrogen. In order to obtain cortical samples for analyses a 1— mm superficial layer was obtained either by trimming the deeper layers away with a cooled burr as described by Schmal *et al.* (1965) or by cleaving the tissue fragments 1—2 mm from the surface with a sharp chisel. In addition, in a few animals cortical tissue from areas covered by bone or tissue from subcortical areas, were sampled. In order to allow a comparison with the freezing method described by Schmal *et al.* (1965) the dura was at full removed in two animals and the tissue was frozen with the method described by these authors.

The rats were anesthetized either with i.p. phenobarbital or with 70 % nitrous oxide and 30 % oxygen. In some rats phenobarbital was given in a dose of 150 mg/kg, in others in a dose of 180—200 mg/kg. In both groups the animals were allowed to breathe room air spontaneously. In the nitrous oxide group, anesthesia was induced with diethyl ether in a closed box. The animals were then tracheotomized, immobilized with curare i.p., and artifi-

cially ventilated with a Palmer miniature respirator. The skull bone was exposed through a longitudinal skin incision, and a plastic funnel was fitted into the incision. The tissue was then frozen by pouring liquid nitrogen into the funnel (Pontén 1966a). With this technique the lung ventilation and the blood pressure were upheld for about 40—70 sec, after which time the animals were removed from the table and frozen in liquid nitrogen for an additional 3—5 min period. The brain was then chiselled out under intermittent irrigation with liquid nitrogen, and the supratentorial parts were saved for analyses. Since it was found that gaseous nitrogen often remained on the table around the tracheotomy cannula, an additional group of animals were analysed in which the nitrogen was blown away with a hairdrier. In order to allow a comparison with previously used freezing techniques (see Discussion), few animals were dropped directly into liquid nitrogen under otherwise identical experimental conditions.

In two rats the local tissue temperature was measured either subdurally or in the cisterna magna during the freezing, using Teflon-coated miniature thermocouple probes and an Electrothermometer (Elektrolaboratoriet, Copenhagen).

In both the cats and the rats arterial blood was anaerobically drawn and analysed for pH, pCO_2 and pO_2 with the help of microelectrodes. CSF was sampled in glass capillaries and analysed for total CO_2. For the analyses of lactate and pyruvate both arterial blood and CSF were allowed to drip directly into liquid nitrogen. The CSF bicarbonate concentration and the pH were calculated as described in previous papers, which also give details of experimental techniques (Pontén 1966a, and b, Pontén and Siesjö 1966, Siesjö and Pontén 1966).

Tissue, CSF and blood samples were stored either at liquid nitrogen temperature or in a low temperature box at −85 °C. Lactate, pyruvate, ATP, ADP, AMP, phosphocreatine and creatine were then measured with specific enzymatic methods (Hohorst, Kreutz and Bücher 1959, Schmal et al. 1965, Bergmeyer 1962). The tissue and the blood samples were crushed in liquid nitrogen, transferred into a glass beaker with ice cold 5 % perchloric acid, rapidly weighed, and homogenized for 15—20 sec with a tissue disintegrator (Ultraturrax, Staufen, Frankfurt a. M.). After one washing and repeated disintegration the combined extracts were centrifuged and the supernatants neutralized with 6 N KOH. All these steps were carried out at 0—4 °C. The neutralization (to pH 4.5—5) was carried out under vigorous stirring and with direct electrometric pH measurement. The subsequent enzymatic measurement of the substrate concentrations were carried out at 340 mμ in a Zeiss PMQ II spectrophotometer with an attached Sargent SRL lin-log recorder. In all cases the entire enzymatic reaction was recorded. The enzymes and the coenzymes used for the analyses were used as commercially obtained (Boehringer, Sigma). The accuracies of the methods used were tested by running standard samples, obtained from the same firms, through the whole procedure.

In blood and CSF only lactate and pyruvate were analysed. For the analyses of lactate and pyruvate in the CSF samples the frozen CSF was allowed to thaw in cold perchloric acid without previous homogenization.

In order to allow a comparison with the extraction technique described by Minard and Davis (1962) who extracted the tissue at −80 °C, the brains of four rats were split into two parts, and one half was extracted at −15 °C as described by Lowry et al. (1961). This is a modified procedure for deproteinizing the tissue below 0 °C which does not necessitate the use of organic solvents.

It is well known that the distribution of weak acids and bases between extra- and intracellular compartments is influenced by differences in pH (Jacobs 1940, Conway and Downey 1950, Milne, Scribner and Crawford 1958). Thus, if only the unionized form of the compound is diffusible and if it exists in equal concentrations in the extra- and intracellular spaces (diffusion equilibrium), the relation between the total concentrations of the acids and their anions in the extra- and intracellular compartments (C_e and C_i) will be given by the equation

$$\frac{C_e}{C_i} = \frac{1+10^{pH_e-pK}}{1+10^{pH_i-pK}} \quad (1)$$

where pK is the ionization constant of the diffusing acid. However, pH is given by the equation

$$pH = pK' + \log\frac{(HCO_3^-)}{P_{CO_2} \cdot S} \quad (2)$$

where pK′ is the apparent ionization constant of carbonic acid and S the solubility factor. If we make the reasonable assumption that P_{CO_2}, pK, pK′ and S are equal in the extra- and intracellular spaces, we can combine equations (1) and (2) and simplify the expression to give

$$\frac{C_e}{C_i} = \frac{(HCO_3^-)_e}{(HCO_3^-)_i} \quad (3)$$

The simplified expression will be valid for acids such as lactic acid and pyruvic acid which has pK values (3.8 and 2.5) which re at least 2 units lower than any expected pH alue

For the calculation of distribution ratios for lactic acid and pyruvic acid the lactate, pyruvate and bicarbonate concentrations in CSF were assumed to represent uniform extracellular concentration. In order to obtain the intracellular concentrations the total tissue concentrations were first corrected for the presence of 3 % blood, using the actual blood concentrations, and then for the presence of 12 % extracellular space using the CSF concentrations. The figure for the size of the extracellular space is an approximation of recent results (Rall, Oppelt and Patlak 1962, Woodward, Reed and Woodbury 1967) The cell lar concentrations obtained were then calculated per unit weight of intracell lar water

The bicarbonate concentrations in rat brain and CSF were derived from the figures published by Pontén (1966 and b) while the corresponding values for cat cortex and CSF ere determined in cats under identical experimental conditions using methods previously described (Siesjö 1962 Pontén and Siesjö 1966) The results of these experiments will be published separately

Results

The results obtained on rat brain tissue are shown in Table I The first three groups represent animals, the brains of which were frozen with a technique devised to give minimal interference with oxygenation (see Methods) It is seen that the various

TABLE I. Lactate, pyruvate, ATP ADP P-creatine and creatine concentrations in rat brain first four groups the brains were frozen *in situ* by pouring liquid nitrogen into plastic tubes t prevent the nitrogen gas from reaching the region of the tracheal cannula. I

Group	Freezing method Anesthesia	P_{CO_2}	Hb	Lact.	Pyr
1.	Present N_2O n = 8	36.2 ±1 1	15.2 ±0 4	1 46 ±0 09	0 105 ±0.011
2.	Present Phenobarb. 175—200 mg/kg = 8	41.2 ±0 8	17.3 ±0.4	0 86 ±0.05	0.076 ±0.004
3.	Present Phenobarb. 150 mg/kg = 8	38.6 ±0.2	16.9 ±0.9	1 12 ±0 13	0 068 ±0 005
4.	Present Phenobarb. 1.6 mg/kg = 7	35 9 ±1 1	17 7 ±0 4	1 90 ±0 32	0.090 ±0 06
5	Phenobarb. 150—180 mg/kg Head dipped into liq. N = 6	39.8 ±0 7	17.8 ±0 4	2 42 ±0 19	0.090 ±0.005

forms of anesthesia gave clear-cut differences in the metabolite concentrations. Thus, a comparison between the groups anesthetized with nitrous oxide and 175—200 mg phenobarbital/kg respectively shows a significantly higher lactate/pyruvate ratio, and a significantly higher lactate concentration in the nitrous oxide group. With a lower phenobarbital dose (150 mg/kg) both the lactate concentration and the lactate/pyruvate ratio increased significantly. However in this group the ATP and the phosphocreatine values were the highest and the ADP values the lowest observed, thus showing a differential effect of the anesthesia on the lactate/pyruvate system, and on the ATP and phosphocreatine concentrations (see Discussion).

The fourth group of Table I shows in comparison with group 3 a much larger variability in results with a significant decrease in the phosphocreatine concentration and in the phosphocreatine/creatine ratio. There was also a tendency towards a higher lactate concentration, and towards a higher lactate/pyruvate ratio. This group thus illustrates the changes obtained when the animals were exposed to an atmosphere with a low oxygen concentration during the freezing. This is seen to a

(mMol/kg wet tissue) P_{ACO_2} in mm Hg and hemoglobin concentration in g %, are given. In the funnel fitted over the exposed skull bone ("present method"). I group 4 no precautions were group 5 the heads of the animals were dipped directly int liquid nitrogen

ATP	ADP	PCr	Cr	Lact./Pyr	ATP/ADP	PCr/Cr
2.34	0.66	3.91	6.03	13.0	3.5	0.65
±0.06	±0.02	±0.10	±0.14	±0.6	±0.2	±0.03
2.43	0.63	4.29	6.26	11.5	3.9	0.69
±0.02	±0.03	±0.06	±0.09	±0.2	±0.2	±0.02
2.54	0.53	5.00	5.55	17.1	4.7	0.91
±0.03	±0.01	±0.05	±0.29	±1.3	±0.1	±0.03
.41	0.60	4.13	6.64	19.6	4.2	0.63
±0.05	±0.03	±0.28	±0.40	±3.3	±0.3	±0.06
2.31	0.77	2.95	7.36	27.2	3.1	0.40
±0.02	±0.02	±0.04	±0.12	±2.3	±0.1	±0.01

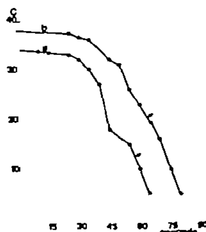


Fig. 1 Fall of local brain temperature subdurally in temporal region ( ) and in cisterna magna (b) during freezing of the rat brain *i* *n* *t* with liquid nitrogen. Respiratory arrest is denoted by arrows.

much more marked degree in the fifth group of animals, the heads of which were submerged in liquid nitrogen. This procedure obviously leads to a marked lactate production, to a substantial breakdown of phosphocreatine and to a significant increase in ADP It was obvious that these metabolic changes occurred during the freezing of the brain. Since the time required to get the central parts of the brain frozen must vary with the size of the animal, a few measurements were made of the local tissue temperature during the freezing (Fig. 1) These measurements showed that there was a good correspondence between the lag between onset of freezing and disappearance of spontaneous ventilation or spontaneous heart activity and the time taken for the medullar regions to reach temperatures close to 0 C.

Table II shows the values obtained on the cortical tissue of cats. It is seen that as compared to the rat brains there was a lower ADP concentration, and thus also higher ATP/ADP ratio. The experiments in which the freezing technique of Schmal et al. were used were too few to allow a comparison between the methods, but the values obtained with our present freezing technique make it obvious that the technique of Schmal *et al.* does not offer any advantages. Comparison between

TABLE II. Metabolite concentrations in cortical tissue from cats in phenobarbital anesthesia (*f*

Freezing method	P_{ACO_2}	Hb	Lact	Pyr	ATP
Frozen = 7	34.1 ±1 7	12.4 ±0.5	0 705 ±0.07	0.063 ±0 007	2.13 ±0 04
Schmal *et al.* (1966) = 2	36.5	11 1	0.844	0.057	1.97

values obtained on cortical tissue from the craniotomy region and tissue from other cortical, or from subcortical, regions did not show any consistent differences.

In Table III the lactate and pyruvate concentrations in CSF and brain tissue have been compared. The results showed that, in general, the lactate and pyruvate concentrations in the CSF were higher than in the intracellular water phase. Thus, since CSF is more alkaline than intracellular water the results lend some support to the assumption of a pH dependent lactate and pyruvate distribution. However the quantitative agreement was equivocal. Table IV shows a comparison between the actual and the theoretically expected distribution (see Methods) In the cat, the lactat distribution approached the theoretical while the pyruvate concentration in the CSF was largely in excess of that predicted. In the rat groups, the CSF lactate concentrations and the CSF lactate/pyruvate ratios were higher than in the cats and the lactate/pyruvate ratios were more in agreement with the corresponding tissue ratios. However an evaluation of the distribution ratios for lactate and pyruvate was made difficult by the pronounced effect of the type of anesthesia upon the tissue lactate concentrations, and thus on the tissue lactate/pyruvate ratio. The rat experiments did not give any clear indication of a differential distribution of lactate and pyruvate although the phenobarbital (but not the nitrous oxide) groups suggested that, in these species, lactate was specifically concentrated in the CSF (see Table IV

Table V show a comparison between the tissue metabolite concentrations in a group of brains extracted either at 0—2° C or at −15 C (see Methods) The group consisted of brains from fou of the animals anesthetized with nitrous oxide and the brains were split longitudinally into two parts before analysis. The results confirmed the results of Lowry *et al.* (1964) in showing a significantly higher phosphocreatine concentration, and a significantly lower ADP concentration in the bra ns extracted at −15 C but they also showed a higher ATP concentration and a much smaller variability of the results, suggesting that part of the ariability normally encountered is due to autolysis during the extraction at temperatures above 0 C. Four additional brains were originally prepared but the tissue was extracted only one time w th perchloric acid at −15 C, a procedure which gav somewhat lower phosphocreatine alues, and somewhat larger lactate/pyruvate ratios.

Table II)

ADP	PCr	Cr	Lact. / Pyr	ATP / ADP	PCr / Cr
0.29	4.80	6.02	11.3	7.6	0.80
±0.01	±0.09	−0.27	±0.4	±0.5	0.03
0.43	3.35	7.0?	15.2	4.9	0.48

TABLE III. Comparison between lactate and pyruvate concentrations and lactate/pyruvate ratios in brain tissue and CSF in various groups of animals. The rat and cat groups are the same as those given in Table I and II. The concentrations are expressed as mEq/kg of wet tissue or CSF

Metabolites	Rats Group 1 Brain	Rats Group 1 CSF	Rats Group 2 Brain	Rats Group 2 CSF	Rats Group 3 Brain	Rats Group 3 CSF	Cats Brain	Cats CSF
Lactate	1.46 ±0.09	2.46 ±0.13	0.86 ±0.03	2.62 ±0.17	1.12 ±0.13	2.99 ±0.19	0.71 ±0.07	1.21 ±0.03
Pyruvate	0.103 ±0.011	0.167 ±0.009	0.076 ±0.004	0.160 ±0.009	0.066 ±0.005	0.160 ±0.009	0.062 ±0.007	0.194 ±0.005
Lact./Pyr	15.0 ±0.6	14.8 ±0.6	11.3 ±0.2	16.8 ±0.4	17.1 ±1.3	18.9 ±0.5	11.3 ±0.4	6.4 ±0.2
n	8	4	8	7	4	4	7	40 (31)

TABLE IV. Comparison between the calculated and experimentally found concentration ratios for lactate and pyruvate between CSF and intracellular water in the brain (see Methods and Table I, II and III)

Group	P_{ACO_2}	HCO Brain	HCO CSF	Lact. Brain	Lact. CSF	Pyr Brain	Pyr CSF	$R_{Lact.}$ Exp	$R_{Lact.}$ Theor	R_{Pyr} Exp.	R_{Pyr} Theor
Cats	34.1	12.0	22.9	0.81	1.22	0.057	0.196	1.51	1.91	3.43	1.91
Rats Group 1	36.2	14.4	27.6	1.70	2.48	0.125	0.17	1.46	1.92	1.31	1.92
Rats Group 2	41.2	15.0	28.6	0.79	2.65	0.084	0.16	3.34	1.91	1.90	1.91
Rats Group 3	36.6	14.5	27.7	1.12	3.02	0.069	0.16	2.70	1.91	2.31	1.91

Discussion

The present experiments were devised to study the relation between the lactate/pyruvate systems in CSF and in brain tissue water. However, during the course of the study it became apparent that some purely methodological questions had to be studied before the main topic could be approached. Thus, both the type of anesthesia used and the freezing method employed had significant effects on the

Table [illegible] Comparison of [illegible] metabolite concentrations obtained by using two different [illegible] methods [illegible]

Extraction temperature	Lac	Pyr	ATP	ADP	PCr	Cr	Lact/Pyr	ATP/ADP	PCr/Cr
[illegible] C			[illegible]	0.[illegible]	[illegible]	[illegible]	14	3	[illegible]
			[illegible]	± [illegible]	±0.15	±0.[illegible]	±1[illegible]	±[illegible]	[illegible]
[illegible] C			[illegible]	0.41	1		1[illegible]9	[illegible]	[illegible]
			[illegible]	± [illegible]	±0.11	±0.16	±0.5	±0.[illegible]	±[illegible]

[illegible] made [illegible] some methodological factors which will be discussed [illegible]

[illegible] experiments have shown that [illegible] nitrous oxide [illegible] cortex [illegible] from which [illegible] lower [illegible] cerebral metabolism and circulation [illegible] higher tissue lactate concentration [illegible] than deep [illegible] with a more superficial [illegible] anaesthesia [illegible] concentrations [illegible] during [illegible] in this case both the phosphocreatine and the ATP [illegible] significantly higher ATP/ADP and [illegible] under nitrous oxide. These results show that [illegible] have differential effects on the lactate/pyruvate ratio [illegible] cytoplasmic redox state [illegible] and utilization of phosphocreatine and ATP. This [illegible] effect than that reported previously [illegible] when anaesthesia were found to cause a decreased lactate [illegible] concentrations in the tissue. It is [illegible] between the effects of [illegible] and phenobarbital [illegible] reported [illegible] et al. 1965 [illegible] differences in action. The observed difference [illegible] of potential importance [illegible] changes. [illegible] phenobarbital [illegible] as a weak acid [illegible] experiments is influenced by pH difference [illegible] experiments [illegible] and Butler 19[illegible] [illegible] shown [illegible] the lactate [illegible] in the brain [illegible] been done.

[illegible] and S[illegible] [illegible] of the brain [illegible] concentrations of labile metabolites [illegible] modification of the [illegible] have been reported. However, small [illegible]

like mice and rats have usually been frozen by direct immersion of the animals in liquid gases (see e g Minard and Davis 1962, Lowry et al. 1964) Richter and Dawson (1948) reported that 9—16 sec were required to freeze the central parts of the brain of a 35 g mouse but, since the local brain temperature was upheld at 37 C during the freezing until it suddenly fell below 0° C, the authors concluded that the circulation of the tissue was upheld during the largest part of the freezing period, preventing such autolytic changes which were seen after decapitation. The present results have essentially confirmed the findings of Richter and Dawson (1948) in showing a rapid terminal fall in the local brain temperature, but they have also shown that these authors, and subsequent experimentators, have paid less attention to another important point. Thus, if an animal is submerged into liquid nitrogen its ventilation will be rapidly inhibited, and it will inspire pure nitrogen before it reaches the point of ventilatory standstill. This is unequivocally shown in Table I which shows, among other things, a substantial lactate production, and a marked breakdown of phosphocreatine in the head-dipped animals. It is clear that the artifacts caused by autolytic changes during the freezing will be inversely proportional to the size of the brains, and they may be virtually absent when mice are being used (cf Lowry et al. 1964) If, however this freezing method is employed in studies in which animals of various sizes are compared, serious errors may be introduced.

The present freezing method (Pontén 1966a) avoids hypoxia during the freezing since it allows free ventilation and continuous heart activity during the freezing The ventilation and the cardiac activity will apparently go on until the freezing front has reached the medullary centers (see Results) Optimal results will then be obtained if the gaseous nitrogen is prevented from reaching a tracheal cannula, which opens to room air and if the liquid nitrogen is prevented from coming close to the medullary centers, especially if the atlanto-occipital membrane is exposed With an adequate freezing technique even deeper regions in such large brains as the cat brain can apparently be successfully studied, and there seems to be no point in using techniques such as the one described by Schmal et al (1965) which also prevents studies of anything but the superficial cortical layers.

Extraction technique The present experiments have confirmed that extraction of the tissue with perchloric acid at temperatures below 0 C gives lower values for ADP and higher values for phosphocreatine (cf Lowry et al 1964 and they have also shown a clear effect on the ATP concentration as well as on the variability of the results The results thus emphasize that great care has to be taken both in freezing and in extracting the tissue if labile metabolites such as ATP ADP AMP P and phosphocreatine are to be studied

Partition of lactate and pyruvate between t[illegible] [illegible] extracellular spaces. There seem to be marked species differences in the CSF lactate pyruvate ratio. Thus, both in cats (present experiments and in dogs Kaasik et al 1968) lactate/pyruvate ratios of 6—7 can be measured while in [illegible] (present experiment) and in humans

TABLE V. Comparison of brain metabolite concentrations obtained by using two different extraction methods (see Methods)

Extraction temperature	Lact.	Pyr	ATP	ADP	PCr	Cr	Lact./Pyr	ATP/ADP	PCr/Cr
0° C.	1.29	0.09	[illegible]5	0.65	3.99	[illegible].91	14.7	3.5	0.68
n = 4	±0.09	±0.00[illegible]	±0.10	±0.0[illegible]	±0.15	±0.09	±1.2	±0.[illegible]	±0.03
−15° C.	1.28	0.0[illegible]1	[illegible].60	0.41	5.15	[illegible]6	1[illegible].9	6.4	0.89
n = 4	±0.10	±0.0[illegible]	±0.01	±0.0[illegible]	±0.11	±0.16	±0.5	±0.4	±0.04

results. A study was therefore made of some methodological factors which will be discussed under separate headings.

Anesthesia. The present experiments have shown that 70 % nitrous oxide-30 % oxygen in combination with curare, an anesthetic form which is known to cause minimal interference with cerebral metabolism and circulation (Wollman *et al.* 1965), is accompanied by a significantly higher tissue lactate concentration, and a significantly higher lactate/pyruvate ratio than deep (150—200 mg/kg) phenobarbital anesthesia. However, with a more superficial barbiturate anesthesia (1.0 mg/kg) the tissue lactate and pyruvate concentrations approach those obtained during nitrous oxide anesthesia, but in this case both the phosphocreatine and the ATP concentrations are increased to give significantly higher ATP/ADP and phosphocreatine/creatine ratios than under nitrous oxide. These results show that the various forms of anesthesia have differential effects on the lactate/pyruvate ratio, which is supposed to reflect the cytoplasmic redox state (see Introduction) and on the balance between the synthesis and utilization of phosphocreatine and ATP. This is thus another anesthetic effect than that reported previously (Stone 1938, Richter and Dawson 1948), when anesthetics were found to cause a decreased lactate and an increased phosphocreatine concentration in the tissue. It is conceivable that some of the differences between the effects of pentobarbital and phenobarbital, as reported by Schmahl *et al.* (1965), may be due to differences in anesthetic concentration, and not to differences in action. The observed differences with various doses of phenobarbital may also be of potential importance in studies of acid-base changes. Thus, since phenobarbital is a weak acid (pK = 6.4), the distribution of which between body compartments is influenced by pH differences between the compartments (Waddell and Butler 19[illegible]7), it must be shown that a given effect of an acid-base change on e.g. the lactate/pyruvate system in the brain is not due to variations in the anesthetic dose.

Freezing method. Ever since Kerr (193[illegible]) and Stone (1938) introduced *in situ* freezing of the brain to preserve *in vivo* concentrations of labile metabolites, various modifications of the freezing procedures have been reported. However, small animals

MINARD, F. N. and R. V. DAVIS, The effects of electroshock on the acid-soluble phosphates of rat brain. *J. biol. Chem.* 1962. 237 1283—1289
PONTÉN, U. Acid-base changes in rat brain tissue during acute respiratory acidosis and baseosis. *Acta physiol. scand.* 1966a. 68 152—163.
PONTÉN, U. Consecutive acid-base changes in blood, brain tissue and cerebrospinal fluid during respiratory acidosis and baseosis. *Acta neurol. scand.* 1966b. 42 455—471
PONTÉN, U. and B. K. SIESJÖ, Gradients of CO_2 tension in the brain. *Acta physiol. scand.* 1966. 67 129—140.
RALL, D. P., W. W. OPPELT and C. S. PATLAK, Extracellular space of brain as determined by diffusion of inulin from the ventricular system. *Life Sciences* 1962. 2 43—48.
RICHTER, D. and R. M. C. DAWSON, Brain metabolism in emotional excitement and in sleep. *Amer. J. Physiol.* 1948. 154 73—79
SCHMAHL, F. W., E. BETZ, H. TALKE and H. J. HOHORST, Energiereiche Phosphate und Metabolite des Energiestoffwechsels in der Grosshirnrinde der Katze. *Biochem. Z.* 1965 342 518—531
SIESJÖ, B. K., The solubility of carbon dioxide in cerebral cortical tissue from cats at 37.5 C. With a note on the solubility of carbon dioxide in water, 0.16 M NaCl and cerebrospinal fluid. *Acta physiol. scand.* 1962. 55 325—341
SIESJÖ, B. K., A. KJÄLLQUIST and N. ZWETNOW, The CSF lactate/pyruvate ratio in cerebral hypoxia. *Life Sciences* 1968. 7 45—52.
SIESJÖ, B. K. and U. PONTÉN, Acid-base changes in the brain in nonrespiratory acidosis and alkalosis. *Exp. Brain Research* 1966. 2 176—190.
STONE, W. E., The effects of anaesthetics and convulsants on the lactic acid content of the brain. *Biochem. J.* 1938. 32 1908—1918.
THORN, W., H. SCHOLL, G. PFLEIDERER and B. MUELDENER, Stoffwechselvorgänge im Gehirn bei normaler und herabgesetzter Körpertemperatur unter ischämischer und anoxischer Belastung. *J. Neurochem.* 1958. 2 150—165.
WADDELL, W. J. and T. C. BUTLER, The distribution and excretion of phenobarbital. *J. clin. Invest.* 1957. 36 1217—1226.
WILLIAMSON, D. H., P. LUND and H. A. KREBS, The redox state of free nicotinamide-adenine dinucleotide in the cytoplasm and mitochondria of rat liver. *Biochem. J.* 1967. 103 514—527
WOLLMAN, H., S. C. ALEXANDER, P. J. COHEN, T. C. SMITH, P. E. CHASE and R. VAN DER MOLEN, Cerebral circulation during general anesthesia and hyperventilation in man—thiopental induction to nitrous oxide and d-tubocurarine. *Anesthesiology* 1965. 26 329—334
WOODWARD, D. L., D. J. REED and D. M. WOODBURY, Extracellular space of rat cerebral cortex. *Amer. J. Physiol.* 1967. 212 367—370.

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Influence of Gonadotrophins on the Respiration of Isolated Cells from the Prepubertal Rat Ovary

By

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Abstract

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A detailed description for separation by microdissection of various types of cells from prepubertal rat ovaries is reported. The rate of oxidation of succinat under influence of gonadotrophins was measured in the various cell samples applying the micro-diver technique (Zeuthen 1 53 The dry weight of the cell samples was determined by direct -ray absorption technique Rosengren 1 59 The gonadotrophins were injected to the animals either hrs before removal of the ovaries or added directly to the incubation medium. I solated granulosa cells the rat of oxygen uptak was significantly enhanced by LH administered *in vivo* or *in vitro* while FSH had no stimulatory effects. The theca cells showed the opposite pattern a stimulatory effect on the rate of oxygen uptak by FSH both *in vivo* and *in vitro* but no stimulatory effect by LH The oxygen uptake of interstitial cells was clearly stimulated by FSH *in vitro* and probably also by *in vitro* administration of this hormone LH showed no tendency to increase the rate of oxygen uptake by these cells. The results support the theory that FSH and LH selectively stimulat various types of ovarian cells

Present knowledge of the action of gonadotrophins on various cell types within the ovary is still very limited Experiments on whole ovaries or sliced tissue have been the main source of information until now It is, however not possible to refer the results of these experiments to a cellular level due to the morphological and functional complexity of this organ. Thus there is clearl a need for methods which make investigations on isolated and separate tissue elements possible.

In histological studies concerned with the prerequisites for oestrogen secretion Falck (1959 described a method for separating various cell types within the rat ovary However in biochemical investigations there is a demand for relatively large amounts of cellular material and hence studies on isolated ovarian cells have been performed on species with large-sized ovaries *e.g* granulosa cells from the pig ovary (Bjersing and Carstensen 1964 1967 and equine ovarian cells (R an and Short 1965, 1966) Recently published reports giving an account of results where various ovarian cells have been grown in tissue culture constitute another promising approach (Channing 1966. Bergman, Bjersing and Nilsson 1966

The majority of work performed on isolated ovarian cells has so far been focused upon problems involving biosynthesis and secretion of steroid hormones. In view of the size of the samples possible to obtain from the rat ovary measurements of gonadotrophic influence on *e g* steroidogenesis in the various cell types are excluded, for methodological reasons. However more general features of the cellular metabolism can reflect the functional state of the cells. Variations in the capacity of protein-synthesizing and energy transducing systems of a cell have been extensively used for various organs as indicators of cellular activities under differing experimental conditions. Measurements of the rate of oxidation of succinate were chosen for the present study thus examining an important and widely applied system which reflects mitochondrial activity within organized cells. Undoubtedly fractionation of cells into separate components may increase the possibilities of extensively characterizing each fraction, but on the other hand the maintenance of the cellular structure is important in study of this type mainly "because the properties of enzymes *in sit* can differ in many ways from those of the pure enzymes and such differences are often of great physiological interest, because they may throw light on the factors which regulate enzyme activity (Krebs 1962)

In a number of previous studies in this laboratory the rates of gl cose uptake and lactic acid production ha e been examined in whole isolated rat ovaries under influence of gonadotrophins either injected to the animals shortly before removal of th ovaries or added directly to the incubation medium (Hamberger and Ahrén 1967a, Ahrén, Hamberger and Hartford 1967 Hamberger Rubinstein and Ahrén 1968) In these studies the effects of an acute *in vivo* administration of gonadotrophins were in principle the same as those obtained by addition of the gonadotrophins *i vitr*

One of the questions of essential importance for this study is the characterization of the different cell types of the ovary with respect to responsiveness to gonadotrophins. A detailed description for separation by microdissection of various types of cells from the prepubertal rat ovary is reported. The oxidation of succinate by various types of cells has been determined by measurements of oxygen consumption with the micro-diver technique of Zeuthen (1953) Acute influences of gonadotrophins ha e been studied, the hormones administered either *in vivo* or *it* A brief summary of the results has been published previously (Hamberger and Ahrén 1967b)

Methods

Animals

Female rats of the Sprague-Dawley strain obtained from A icumex Ltd (Stockholm) or from ow breeding were used. The animals ranged in age betwee 2 nd 36 days and the exact age was know for every rat in all the experiments They were fed semisynthetic diet (Gustavsson 1939) and water *ad libit m*. The effect of starvin he animal for 20—24 hrs before the experiment was systematically studied in comparison h unstarved animals but no differenc between starved and unstarved animals as recorded on he parameters studied. For such reasons the majori y of the rats in this study ha not been starved since starv tion *per se* may induce hanges of the metabolic homeostasis

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From the Department of Physiology University of Göteborg Sweden

Influence of Gonadotrophins on the Respiration of Isolated Cells from the Prepubertal Rat Ovary

By

LARS A. HAMBERGER

Received 1 March 1968

Abstract

HAMBERGER, L. A. *Influence of gonadotrophins on the respiration of isolated c lls from the pr pubertal rat ovary* Acta physiol scand. 1968 74 410—425.

A detailed description for separation by microdissection of various types of cells from pre pubertal rat ovaries is reported. The rat of oxidation of succinate under influence of gonadotrophins was measured in the various cell samples pplying the micro-diver technique (Zeuthen 1953) The dry weight of the cell samples was determined by direct -ray absorption technique (Rosengren 1959) The gonadotrophins were injected to the animals either 2 hrs before removal of the ovaries, or dded directly to the incubation medium. In isolated granulosa cells the rate of oxygen ptake was significantly enhanced by LH administered in vit or i vo while FSH had no stimulatory effects. The theca cells showed the opposite pattern i a stimulatory effect on the rate of oxygen ptak by FSH both vitro and i but no stimulatory effect by LH. The oxygen ptake of interstitial cells was clearly stimulated by FSH vit and probably also by in tr administration of this hormone LH showed no tendency to increase the rate of oxygen ptake by these cells. The results support the theory that FSH and LH selectively stimulat arious types of ovarian cells.

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separate series of experiments the sizes of the cell samples were deliberately ried between 5—150 × 10 mg The mass determinations were further checked using modified pparatus for x-ray absorption with another reference system for the masses (Hagberg, Haljamäe and Röckert 1967) Ten theca cell samples were measured in this apparatus giving mean value 29.±5.1 × 10 mg The corresponding mean alue using Rosengren (1959) original p-paratus was 28.1±5. × 10 mg.

Analytical methods

The oxygen uptake of the various cell samples was determined by the micro-diver technique (Zeuthen 1953 Zajicek and Zeuthen 1961) The diver made of Pyrex glass was fastened m glass handle by means of rubber stopper as described by Brzin and Zeuthen (1961) The open tip of the diver was then introduced into the incubation medium in the chilled glass cup containing the respective cell samples. Under the stereomicroscope, one of these samples was drawn up into the diver by suction together with pproximately 0.3—0.5 μl medium, after which the tip of the diver was sealed with beeswax heated to its mel ing point (around 60 C) The diver was then detached from the handle and adjusted to flotation equilibrium in 0.1 M phosphate buffer pH 7.4 and transferred to flotation essel. The time interval be tween sacrifice of the animals and the introduction of the cell samples m the micro-divers did not exceed 30 min.

The divers were 15—20 mm long, weighing 0.2—0.6 mg for determinations on granulosa cells, and 0.6—1.6 mg for determinations on theca and interstitial cells. The ratio of the weight of the glass used for the divers to the volume of air required to keep the glass afloat in the medium was determined for every sample of glass tubing.

The flotation essels were filled with the same phosphate buffer as that used for the adjustments of the divers. The vessels were immersed in thermostatically regulated water bath with extremely good isolation. The temperature variations measured by thermistor thermometer placed inside the flotation vessels remained below ±0.0015 C. All measurements were per formed at 37 C and eight divers were run simultaneously

After temperature equilibration period of 30—40 min, the oxygen consumption was measured manometrically for 2—3 hrs. The manometric readings of the equilibrium pressure were made every 10—20 min for each diver The pressure was plotted against time and was shown to remain linear for at least 3—4 hrs. Blank divers containing incubation medium but no cells showed no oxygen consumption.

Oxygen uptake was measured in the magnitude of 10 —10 μl/hr and calculated according to Zeuthen (1953) I theoretical calculations and also experimentally Zajicek and Zeuthen (1961) found the error of the micro-diver technique to be ±5 % in determinations of gas consumption or production in the order of magnitude of 10 μl/hr

"T o-compartment divers"

This special technique makes it possible to determine oxygen consumption of the same sample of cells before and after addition of the respectiv gonadotrophic hormones The technique has been described in detail in previous paper (Hamberger 1968

Chemicals

Incubation medium. Na_2HPO_4-KH_2PO buffer pH 7.4 37.5 mM cytochrome 8.6 × 10 mM \ succinate 25 mM $AlCl_3$ 0.5 mM $MgCl_2$ 0.5 mM Slater 1949 Potter 1957) Cytochrome (type III, Sigma Comp.) was dissolved in distilled H_2O immediately before the start of each experiment.

Hormone Bovine luteinizing hormone (NIH LH B5) and ovine follicle stimulating hor mone (NIH FSH S2) were supplied by he Endocrinology Study Section, National Institutes of Health, USA. The hormones were dissolved in 0.9 % NaCl 0.5 mg/ml for the injections, control rats were injected with 0.9 NaCl. The hormones were dissolved in phosphate buffer to gi final concentration of 50 μg/ml for the in itr experiments

Statistical analysis

Mean alues are given ± standard error of the mean S.E.M.) Comparisons between different groups ere performed according to Student' t-test. Both comparisons of all control alues to all experimental values (number of divers) and comparisons of mean ontrol to mean ex perimental alues from each animal were performed. A p-value of 0.05 or less was considered significant.

Results

G anulosa c lls

Eff ts of gonadot phins n cel Oxygen uptake by isol d granulosa cells ob-

TABLE I. Effects of LH and FSH on the oxygen uptake of isolated granulosa cells obtained from 25—30 day old rats.

Administration of hormone	Number of rats	Number of determinations	O_2 uptake[a] (μl/mg × hr)	Significance of hormone effect
In vitro				
Control medium	7	15	33.6±4.3	p<0.001
LH (50 μg/ml)	7	17	81.4±7.6	
Control medium	6	15	37.6±3.5	N.S.
FSH (50 μg/ml)	6	14	32.4±2.6	
In vivo				
Saline	6	16	30.6±3.5	0<0.005
LH (100 μg/100 g b.w.)	6	15	53.6±5.9	
Saline	6	17	41.3±4.4	N.S.
FSH (100 μg/100 g b.w.)	6	17	36.8±2.9	

Bovine LH (NIH-LH-B3) or ovine FSH (NIH FSH-S2) was added either directly to the incubation medium to give a final hormonal concentration of 50 μg/ml or injected in one i. dose (100 μg/100 g b.w.) 2 hrs before removal of the ovaries. (Control rats injected with 0.9 % NaCl). The granulosa cell samples were taken from the same follicle for each *in vitro* experiment and from follicles of comparable size for the *in vivo* experiments. All the cell samples were incubated in microdivers 37° C in medium containing succinate and the rates of oxygen uptake determined. [a] Each granulosa cell sample contained 50 granulosa cells and mass determination by direct -ray technique (Rosengren 1959) performed on 27 different granulosa cell samples gave mean value of $10.15 \pm 1.1 \times 10^{-6}$ mg per 50 granulosa cells. The oxygen uptake is expressed as μl/mg dry weight and hr. Mean values of the number of determinations are given ± S.E.M. (N.S. = Not significant)

tained from prepubertal rat ovaries was studied in medium containing succinate as substrate. In all experiments the rate of oxygen uptake (expressed as μl/mg and hr) in the granulosa cells was raised when LH (50 μg/ml) was added to the incubation medium (Table I). The difference in oxygen uptake between the control cells and the LH stimulated cells was highly significant ($p<0.001$). In contrast, addition of FSH (50 μg/ml) to the incubation medium was without measurable effect on the oxygen uptake of these cells (Table I). In each experiment control and experimental cell samples, 2—3 samples of each type were taken from the same follicle and incubated simultaneously. In a previous series of experiments it was shown (Ahrén, Hamberger and Hamberger 1965) that addition of ACTH or growth hormone to the medium in approximately the same molar concentrations as for LH did not influence the rate of oxygen uptake in the granulosa cells.

The endogenous respiration was investigated in medium without succinate. In these experiments no measurable oxygen consumption of the granulosa cells was found whether the medium contained gonadotrophins or no hormone at all. The age of the rats in these experiments ranged between 25—30 days. In experiments per-

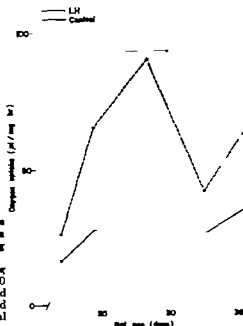


Fig. 1 Influence of age on the responsiveness of isolated granulosa cells to LH. Granulosa cell samples were obtained from rats varying in age between 22—36 days. Oxygen uptake was determined at 37 C in medium containing succinate with or without addition of LH (50 μg/ml) A peak in sensitivity to LH was found at around 29 days of age. The lines are based upon 56 determinations with cellular material from 14 rats.

formed on granulosa cells obtained from rats varying in age between 22—36 days. it was found that maximal stimulation by LH of oxygen uptake was elicited when the rats were around 29 days old (Fig 1)

In studies applying the "two-compartment diver technique (Hamberger 1968) 15 cell samples from 10 rats were run. With this technique the oxygen uptake of the same sample of granulosa cells could be measured before and after addition of gonadotrophins. In all the experiments, there was an increase in oxygen uptake after addition of LH in a final concentration varying between 30—70 μg/ml measurable within 10—15 min with a steady state occurring promptly after the addition of the hormone The average increase in oxygen uptake above the control level was 185 % (Fig 2) Addition of FSH in the same final concentrations as for LH to granulosa cells obtained from the same rat ovaries caused no significant change in oxygen uptake (Fig 2)

Effects of gonadotrophins in vivo In other experiments LH or FSH was injected to 25—30 day old rats in one iv dose (100 μg/100 g b w) hrs before removal of the ovaries. Control rats were injected with 0.9 % NaCl and all rats were under light ether anesthesia during the injections. Granulosa cell samples obtained from control and hormone injected rats were isolated from follicles of comparable size. The cell samples were incubated in the micro-divers and the oxygen uptake was determined. The cell samples from the LH injected rats showed a significantly higher oxygen uptake ($p<0.005$) than simultaneously incubated granulosa cells from control rats, while injections of FSH did not change the rate of oxygen uptake by these cells (Table I)

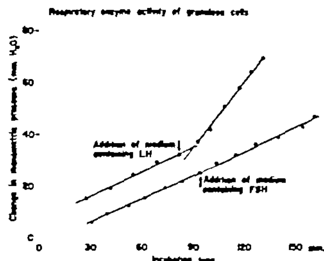


Fig. — Gonadotrophic influence in vitro on the oxygen uptake of isolated granulosa cells obtained from prepubertal rat ovaries. Experiments performed with the "two-compartment diver" (Hamberger 1968). The cells were incubated at 37 C in medium containing succinate. The figure shows two different experiments where the oxygen uptake (expressed as change in manometric pressure) is plotted against incubation time. The first 30 min of the incubation period were used for thermoequilibration. Manometric readings were then performed. Addition of LH (upper curve) in a final concentration of 40—60 µg/ml increased the respiratory rate of the granulosa cells by 155 % above the control level within 10—15 min, while addition of FSH (lower curve) in the same amounts as for LH did not change the rate of oxygen uptake during the incubation period.

Theca cells

Effects of gonadotrophins in vitro. Isolated theca cell samples were incubated in the absence and presence of gonadotrophins. Control and experimental cell samples in each experiment were taken from the same follicular wall and incubated simultaneously. Addition of LH (50 µg/ml) in the same concentration as used in the experiments with the granulosa cells had no stimulatory effect upon the oxygen consumption of isolated theca cells (Table II). FSH on the other hand showed a slight but significant stimulatory effect upon the oxygen consumption of these cells when added to the incubation medium in a concentration of 50 µg/ml (Table II).

In experiments applying the "two-compartment diver" technique (Hamberger 1968) the oxygen uptake by theca cell samples isolated from ovaries obtained from 5 rats (25—30 days old) was measured before and after addition of FSH in a final concentration varying between 50—90 µg/ml medium. In 8 of 9 determinations there was an increase in oxygen uptake measurable within 15 min, with a steady state occurring promptly after the addition of FSH. The average increase above the control level was 72 % (Fig. 3). Addition of LH in the same final concentrations had no stimulatory effect upon the rate of oxygen uptake by these cells.

The endogenous respiration determined in medium without succinate was very low varying between 0.5—2 µl O_2/mg and hr.

Effects by gonadotrophins in vivo. The influence by in vivo administration of LH or FSH on the respiration of theca cells has also been studied and the results are summarized in Table II. Prepubertal rats varying in age between 25—30 days were injected with LH or FSH in one i. v. dose (100 µg/100 g b.w.) 2 hrs before removal of the ovaries. Control rats were injected with 0.9 % NaCl and all injections were performed under light ether anaesthesia. In each experiment theca cell samples from

TABLE II. Effects of LH and FSH on the oxygen uptake of isolated theca cells obtained from 25—30 day old rats.

Administration of hormone	Number of rats	Number of determinations	O_2-uptake[1] (µl/mg × hr)	Significance of hormonal effect
In vitro				
Control medium	5	17	27.1 ± 3.6	N.S.
LH (50 µg/ml)	5	16	22.4 ± 2.0	
Control medium	8	16	19.7 ± 1.7	p < 0.005
FSH (50 µg/ml)	8	19	29.1 ± 2.3	
In vivo				
Saline	5	17	27.1 ± 3.6	N.S.
LH (100 µg/100 g b.w.)	5	16	22.4 ± 2.0	
Saline	7	15	23.4 ± 3.7	p < 0.02
FSH (100 µg/100 g b.w.)	7	16	44.0 ± 6.6	

Bovine LH (NIH LH B3) or ovine FSH (NIH-FSH-S2) was added either directly to the incubation medium to give a final hormonal concentration of 50 µg/ml or injected in one i.p. dose (100 µg/100 g b.w.) 2 hrs before removal of the ovaries. (Control rats injected with 0.9 % NaCl). The theca cell samples were taken from the same follicular wall for each in vitro experiment and from follicles of comparable size for the in vivo experiments. All the cell samples were incubated in micro-divers at 37° C in medium containing succinate and the rates of oxygen uptake determined.

[1] Mass determination was performed on each cell sample subsequent to the incubations by direct X-ray absorption technique (Rosengren 1959). The oxygen uptake is expressed in µl/mg dry weight and hr. Mean values of the number of determinations are given ± S.E.M. (N.S. = Not significant).

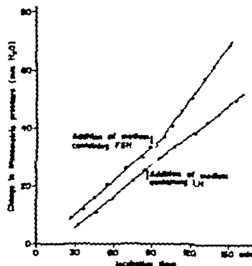
experimental and control rats were collected from follicles of comparable size and run simultaneously. There was an increased rate in oxygen uptake by theca cells obtained from rats injected with FSH compared to both control rats and rats injected with LH. No stimulatory effect was found by LH under these experimental conditions (Table II).

Interstitial cells

Effects of gonadotrophins in vitro. Interstitial cell samples obtained from prepubertal rats varying in age between 25—30 days were incubated in the absence and presence of gonadotrophins. LH and FSH were added to the incubation medium in a final concentration of 50 µg/ml. In each experiment both control and experimental cell samples were taken from the same interstitial mass. FSH *in vitro* caused a slight but insignificant increase in oxygen uptake of these cells, while LH showed no tendency to an influence of this parameter (Table III).

The endogenous respiration determined in medium without succinate was low, not exceeding 2 µl O_2/mg and hr.

In order to further analyse if it was possible to elicit a significant stimulatory effect



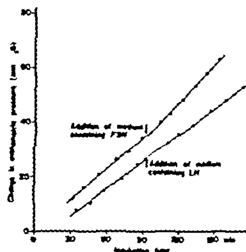


Fig. 3 Influence of gonadotrophins *in vitro* on the rate of oxygen uptake of theca cells isolated from prepubertal rat ovaries. Experiments performed with the two-compartment diver (Hamberger 1968) The cells were incubated at 37 C in medium containing succinate. The figure shows two different experiments where oxygen uptake (expressed as change in manometric pressure) is plotted against incubation time. After 30 min of thermoequilibration the manometric readings were performed. Addition of FSH (upper curve) in final concentration of 70—90 µg/ml increased the respiratory rate of the theca cells by 61 % above the control level while no stimulatory effect was elicited with LH (lower curve) added in the same amount as for FSH

Fig. 4 Influence of gonadotrophins *in vitro* on the rate of oxygen uptake of isolated samples of interstitial cells obtained from prepubertal rat ovaries. Experiments performed with the "two-compartment diver (Hamberger 1968) The cells were incubated at 37 C in medium containing succinate. The figure shows two different experiments where oxygen uptake (expressed as change in manometric pressure) is plotted against time. After 30 min of thermoequilibration manometric readings were performed every 10—15 min for about 2 hrs. Addition of FSH (upper curve) in final concentration of 70—90 µg/ml increased the respiratory rate by 23 % above the control level while LH (lower curve) added in the same amount as for FSH caused no influence on the rate of oxygen uptake by the interstitial cells.

by FSH *in vitro* on the interstitial cells another series of experiments was performed applying the two-compartment diver technique (Hamberger 1968) In 10 determinations from 4 rats, FSH in concentrations varying between 50—100 µg/ml caused a stimulatory effect in 9 instances while no effect was found in one instance. The mean increase expressed in per cent above the control level was 22 (Fig. 4)

Effects of gonadotrophins in vivo In one series of experiments, interstitial cell samples were collected from rats (21—31 days old) injected i.v. with FSH or LH (100 µg/100 g b.w.) 2 hrs before removal of the ovaries. Control rats were injected with 0.9 % NaCl and all injections were performed under light ether anaesthesia. The interstitial cells were incubated in medium containing succinate, and oxygen uptake was measured. The respiratory rate of the interstitial cells was significantly increased by an acute injection of FSH ($p < 0.02$) Administration of LH *in vivo* was, however without stimulatory effect on the oxygen uptake of these cells (Table III)

TABLE III. Effects of LH and FSH on the oxygen uptake of isolated interstitial cells obtained from 25—31 day old rats.

Administration of hormone	Number of rats	Number of determinations	O_2-uptake* ($\mu l/mg \times hr$)	Significance of hormonal effect
In vitro				
Control medium	13	32	21.8±1.7	N.S.
LH (50 μg/ml)	13	34	19.8±1.4	
Control medium	7	16	19.4±2.9	N.S.
FSH (50 μg/ml)	7	17	27.6±4.6	
In vivo				
Saline	7	23	28.6±2.6	N.S.
LH (100 μg/100 g b.w.)	7	18	27.7±2.6	
Saline	7	17	28.6±4.5	p<0.02
FSH (100 μg/100 g b.w.)	7	16	49.7±7.2	

Bovine LH (NIH-LH-B3) or ovine FSH (NIH FSH-S2) was added either directly to the incubation medium to give a final hormonal concentration of 50 μg/ml or injected in one i. p. dose (100 μg/100 g b.w.) 2 hrs before removal of the ovaries. (Control rats injected with 0.9 % NaCl). The interstitial cell samples were taken from the same interstitial gland lobe for each *in vitro* experiment. The cell samples were incubated in micro-divers at 37° C in medium containing succinate and the rates of oxygen uptake determined.

Mass determination was performed on each cell sample subsequent to the incubations by direct x-ray absorption techniques (Rosengren 1959). The oxygen uptake is expressed in μl/mg dry weight and hr. Mean values of the number of determinations are given ± S.E.M. (N.S. = Not significant.)

Discussion

Methods for isolation of various types of cells within the rat ovary by freehand dissection have been described previously by Falck (1959) and in this laboratory (Ahrén, Hamberger and Hamberger 1965) but in the present study a further extension has been made which opens possibilities for measurements on multiple cell samples derived from one and the same follicle under various experimental conditions. It could be argued that the cell populations used were not absolutely pure but within the limitations of the technique and microscopic methods used, it can be concluded that the cells in each sample were predominantly granulosa, theca or interstitial cells. The size of the follicles used, ranging in diameter between 150—500 μ corresponds well to stage 5 and 6 in Mandl and Zuckerman's (1952) classification of follicles. Histological sections revealed that these follicles all contained multiple layers of granulosa cells with or without antra. No difference in respiration was found between cells from smaller or bigger follicles within this range.

In order to obtain quantitative values of the respiratory rates in the separate cells it was necessary to determine the cell mass of the samples utilized for these measurements. Different methods for mass determination have been described, e.g. the Lo

weighing method (Lowry 1941) interference microscopy (Barer and Dick 1957) and microradiography (Lindström 1955) The direct x ray absorption technique (Rosengren 1959) seemed, however to be the most suitable technique for the present purpose. The total error in mass determination with this technique is of the order of ±5 per cent.

The purity of the hormones used (NIH LH B3 and NIH FSH S2) was relatively high but a low degree of contamination by LH in the FSH preparation or *vice versa* cannot be excluded. However for evaluation of the results this was of minor importance in the present study The hormones have been administered in relatively high amounts which have been shown to stimulate other biochemical reactions in the prepubertal rat ovary both *in vitro* and *in vivo* (Ahrén, Hamberger and Hartford 1967 Hamberger and Ahrén 1967a, Ahrén Hamberger and Perklev 1968)

In the above mentioned studies by Falck (1959) the purpose was to investigate which ovarian cells were responsible for oestrogen secretion. This was indirectly investigated by studying changes in the vaginal mucosa which was transplanted to the eye together with ovarian cell types in various combinations. It was demonstrated that the ovarian transplants must contain at least two of the cell types—granulosa or corpus luteum cells in combination with theca or interstitial cells—in order to secrete oestrogen.

Measurements of the rate of oxidation of succinate in various types of ovarian cells was chosen in the present study The use of succinate as substrate deserves some comments. The citric acid cycle is an important source of energy for the ovarian cells and it is possible that gonadotrophic influence can be reflected in various steps of the cycle In 1960 Behrman and Duboff (1960) in experiments performed on human ovaries, demonstrated great variations in the concentrations of the intermediary metabolites of the citric acid cycle during the various phases of the menstrual cycle with a peak in the concentrations for nearly all the metabolites corresponding to the time of ovulation Many years earlier Meyer *et al.* (1945 1947) found that the succinic dehydrogenase activity in the mature rat ovary showed great variations during pregnancy and lactation. and could in addition be enhanced by injections of gonadotrophins. Eckstein and Landsberg (1963) also reported increased activities of isocitric, succinic and malic dehydrogenases in mature rat ovaries as a result of pregnant mare serum gonadotrophin (PMS) injections for 5 days. The situation for the immature rat ovary was, however more complicated. Here too, Eckstein and Landsberg (1963) found increased activities of isocitric and malic dehydrogenases by PMS but no influence on the activity of succinic dehydrogenase On the other hand, Eckstein (1962) reported dramatic increases in succinic dehydrogenase activities, estimated by the tetrazolium reaction method of Kun and Abood (1949) in immature rat ovaries after injections of PMS or human chorionic gonadotrophin (HCG) However in the above mentioned investigations homogenized ovaries, including all the various cell types, were used, and the determinations performed after a minimum of 5 days of gonadotrophic stimulation. A chronic gonadotrophic stimulation of this type is followed by histological changes and a marked weight increase

TABLE IV. Summary of results concerning oxygen uptake in various cell types isolated from prepubertal rat ovaries under the influence of LH or FSH. The hormones were either added directly to the incubation medium (*in vitro*) or injected in one i.v. dose 2 hrs before removal of the ovaries (*in vivo*). Symbols: + = stimulatory effect; — = no effect.

		Granulosa cells	Theca cells	Interstitial cells
LH	*in vitro*	+	—	—
	in vivo	+	—	—
FSH	*in vitro*	—	+	(+)
	in vivo	—	+	+

of the whole ovary. Further, the capacity for steroid production and secretion may be markedly changed. For such reasons, special difficulties arise in the evaluation of gonadotrophic effects on certain enzymatic steps.

In contrast to the above-mentioned investigations, the present study is concerned with acute effects of gonadotrophins administered either by an i.v. injection shortly before removal of the ovaries or by addition of the hormones *in vitro*. Even under these experimental conditions it is, however, important to keep in mind that the results may be influenced by a number of secondary factors in the *in vivo* experiments; on the other hand, the target organ may lack some factors *in vitro* of importance for the hormonal response. It is thus not possible to conclude that a hormonal effect obtained both *in vivo* and *in vitro* is due to the same mechanism, although it is of interest that an effect obtained *in vitro* can also be demonstrated when the hormone is given *in vivo*.

The results of this study are schematically summarized in Table IV. In experiments performed on granulosa cells it was demonstrated that LH administered either *in vivo* or *in vitro* increased the rate of oxygen uptake by these cells. The stimulatory effect by LH *in vitro* was more pronounced in granulosa cells obtained from rats varying in age between 25—30 days, than in cells obtained from younger or older rats (see Fig. 1). The interval between 20—35 days of age has been shown to be eventful from a hormonal point of view in the immature rat. Zarrow and Wilson (1961) reported that the follicles became sensitive in their response to gonadotrophic stimulation at approximately 18 days of age and reached a maximum in responsiveness between 23—26 days of age in studies concerning gonadotrophic induced superovulation. Ovarian responsiveness to exogenous gonadotrophins measured by weight increase of the ovary and uterus was also shown to be maximal around 28—30 days of age (Price and Ortiz 1944, Soper and Ladman 1962). Measurements of pituitary content of LH showed high levels of this gonadotrophic hormone in pituitaries from rats varying in age between 26—32 days followed by a drastic decline indicating a release of the hormone into the circulation (Ramirez and McCann 1963, Moore 1965). It is possible that such a gonadotrophic release can cause a decrease in the

sensitivity to LH *in vitro*. In this connection it is of interest to note that a decreased sensitivity to growth hormone *in vitro* with increasing plasma concentrations of this hormone has been reported in transport studies performed on the rat diaphragm (Hjalmarson and Ahrén 1967). Such a phenomenon may, in fact, reflect a more general mechanism for hormonal influence on various metabolic steps.

Administration of FSH, either *in vitro* or *in vivo* did not influence the rate of oxygen uptake by the granulosa cells (Table IV). The amount of FSH used in these experiments has been shown to stimulate other parameters within the prepubertal rat ovary. Hence, it has been reported that FSH *in vitro* stimulates both glucose uptake and lactic acid production by isolated prepubertal rat ovaries in concentrations varying between 10—1000 μg/ml medium (Hamberger and Ahrén 1967a) and that FSH *in vivo*, in addition, increases the transport rate of amino acids even in lower doses than those used here (Ahrén, Hamberger and Hartford 1967). Since only one preparation of FSH has been used thus far, it may, however, be unwise to draw too wide conclusions from negative results. Nevertheless it can be concluded that a striking difference in responsiveness exists between LH and FSH as far as the granulosa cells are concerned (Table IV).

Compared to the studies on granulosa cells, an opposite pattern of gonadotrophic influence was found in experiments with theca cells, i.e. a stimulatory effect on the rate of oxygen uptake by FSH both *in vivo* and *in vitro*, and a lack of effect by LH administered either *in vitro* or *in vivo* (see Table IV). The effect by FSH *in vitro* was of special interest since it recently has been shown that this hormone was capable of increasing the rate of glucose uptake by whole isolated prepubertal rat ovaries when added directly to the incubation medium (Hamberger and Ahrén 1967a).

In studies using autoradiographic technique, it has been shown that an acute injection of FSH stimulated the incorporation of labelled amino acids selectively into the theca cells of prepubertal rat ovaries, but showed no detectable influence on the granulosa cells (Rubinstein 1967, Hamberger, Sjöstrand and Ahrén 1968). Again, cautiousness in the evaluation of the negative results with LH must naturally be taken. However, the findings that FSH and LH have distinct and separate effects on the metabolism of granulosa and theca cells in follicles from prepubertal rat ovaries in the present study is of interest in relation to the above-mentioned "two cell type" theory for oestrogen secretion, which has been examined and discussed by a number of investigators (Westman 1929, 1934, Falck 1959, Bjersing and Carstensen 1964, Ryan and Short 1966). Further, Fevold (1941) and Greep *et al.* (194) reported that injections of FSH alone to hypophysectomized rats did not stimulate oestrogen secretion. This observation has been confirmed and extended in recent studies by Lostroh and Johnson (1966). The last mentioned investigators injected highly purified preparations of FSH and LH separately or in combination to hypophysectomized rats. They found that, injected alone, neither of these gonadotrophins stimulated the release of sufficient amounts of oestrogen to cause uterine development. When, however, the two gonadotrophins were injected in combination, there was a marked secretion of oestrogen. From these studies a combined action of FSH

and LH seems to be necessary for induction of oestrogen secretion in the rat ovary

The controversial problem of the functional significance of the interstitial cells as well as their origin has been discussed for many years and is not yet settled Rennels (1951) and Dawson and McCabe (1951) reported that the interstitial tissue of the rat ovary was of dual origin, one primary type closely associated with granulosa cell outgrowths and ingrowing cords from the germinal epithelium, and one secondary type formed from the theca interna of atretic follicles. Studies concerning the function of the interstitial cells indicate that these cells are involved in the ovarian production of both androgens and oestrogens (Claesson 1954 Falck 1959 Harrison 1962) Interstitial cells can be histochemically identified by the Schultz or Schiff reactions (Rennels 1949 1951) The reactive materials in these reactions reach a maximal distribution around the 15th day of life, and high concentrations persist until the first ovulation.

In the present investigation, the reactivity to gonadotrophins was very similar in the interstitial cells and in the theca cells i.e. a marked stimulatory effect by FSH, at least when administered *in vivo* and no influence at all by LH neither *in vitro* nor *in vivo* (Table IV) This similarity in reactivity between theca and interstitial cells fits with Falck studies concerning steroid secretion in the rat ovary As mentioned above, theca *or* interstitial cells were necessary for oestrogen secretion (Falck 1959) After hypophysectomy the interstitial cells progressively transform into the deficiency cell type, but respond to pituitary extracts (Selye Collip and Thomson 1933) or to gonadotrophins extracted from human urine (Lyon Simpson and Evans 1953) by again assuming the appearance of typical interstitial cells. Claesson and co-workers (Claesson *et l* 1953 Claesson 1954) made an extensive investigation of the lipid content, mainly of the cholesterol fraction, in interstitial cells from the rabbit ovary They demonstrated that injections of PMS markedly and rapidly (within 2 hrs) decreased the cholesterol content of these cells. Recently it was reported (Shilno and Rennels 1967) that a highly purified HCG preparation caused enlargement of the interstitial cells in immature rat ovaries. Although it has been shown previously (g Hamberger and Ahrén 1967a) that HCG and LH can be very similar in their reactions, they re not identical hormones. In summary several investigators have demonstrated that interstitial cells are dependent upon gonadotrophic stimulation for their development and function nd that gonadotrophic hormone preparations with considerable FSH-like ctivity can influence these cells

The results from the present study upport the theory f selective gonadotrophic regulation of metabolic steps in certai types of ovarian cells Experiments concerned with gonadotrophic influence on corpus l teum cell and oocytes applying the microdiver technique (Zeuthen 1953) are now progres

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References

Ahrén K. E. B A. C. Hamberger and L. A. Hamberger, Action of luteinizing hormone in vitro on respiratory enzyme activity of isolated granulosa cells. *Endocrinology* 1965 *77* 332—336

Ahrén K., L. Hamberger and M. Hartford Further studies on the effect of follicle stimulating hormone on amino acid transport in the isolated rat ovary *Acta physiol. scand.* 1967 *71* 211—223

Ahrén, K., L. Hamberger and T Perklev Effects of human menopausal gonadotropin and ovine follicle stimulating hormone on lactic acid production by ovaries from prepubertal rats. *Endocrinology* 1968. *82* 420—422.

Ahrén, K., L. Hamberger and L. Rubinstein Influence of puromycin and anoxia on the effect of gonadotrophins on ovarian carbohydrate metabolism. *Acta physiol. scand* 1968 *74* 79—90.

Barer, R. and D A. T Dick, Interferometry and refractometry of cells in tissue culture. *Exp Cell Res.* 1957 Suppl. 4 103—135.

Behrman S J and G. S Duboff Tricarboxylic acid metabolism studies in the ovary throughout the menstrual cycle. *Fertil. and Steril* 1960. *11* 210—220.

Bengtsson S., L. Bjersing and O. Nilsson Histochemical demonstration of Δ 5,3β-hydroxysteroid dehydrogenase activity in cultivated granulosa cells of the porcine ovary *Acta path. microbiol. scand* 1966. *68* 461—46.

Bjersing, L. and H. Carstensen The role of the granulosa cell in the biosynthesis of ovarian steroid hormones. *Biochim. biophys. Acta* 1964 *86* 639—640

Bjersing. L. and H. Carstensen. Biosynthesis of steroids by granulosa cells of the porcine ovary in vitro *J Reprod Fertil.* 1967 *14* 101—111

Brzin M and E. Zeuthen. Quantitative evaluation of the thiocholine method for cholinesterase as applied to single end-plates from mouse gastrocnemius muscle *C R. Lab Carlsberg* 1961 *32* 139—153

Channing C P Progesterone biosynthesis by equine granulosa cells growing in tissue culture. *Nature* (Lond.) 1966. *210* 1266.

Claesson L Quantitative relationship between gonadotrophic stimulation and lipid changes in the interstitial gland of the rabbit ovary *Acta physiol. scand.* 1954 31 Suppl. *113* 23—51

Claesson, L., N A. Hillarp and B Högberg. Lipid changes in the interstitial gland of the rabbit ovary at oestrogen formation. *Acta physiol scand.* 1953 *29* 329—339

Dawson A B and M McCabe. The interstitial tissue of the ovary in infantile and juvenile rats. *J Morph* 1951 *88* 543—571

Eckstein B The development of the ovary of the rat II. *Acta endocr* (Kbh.) 1962 *41* 35—40

Eckstein, B and R Landsberg Effect of serum gonadotrophin on dehydrogenases of the citric acid cycle in the ovary of the rat. *Acta endoc* (Kbh.) 1963. *42* 480—484

Falck. B., Site of production of oestrogen in rat ovary as studied in microtransplants. *Acta physiol. scand* 1959 *47* Suppl. 163 1—101

Fevold H. L Synergism of follicle stimulating and luteinizing hormones in producing estrogen secretion. *Endocrinology* 1941 *28* 33—36.

Greep R. O H B Van Dyke and B F Chow Gonadotrophins of swine pituitary various biological effects of purified thylakentrin FSH and pure metakentrin (ICSH) *Endocrinology* 1942. *30* 635—649

Gustafsson. B. E Lightweight stainless steel systems for rearing germfree rats. *Ann. N.Y Acad Sci.* 1959 *78* 17

Hagberg, S., H. Haljamäe and H Röckert Shock reactions in skeletal muscle, II *Acta chir scand* 1967 *133* 265—268

Hamberger. L. A., A "two-compartment diver" in the micro-diver technique. *Acta physiol. scand* 1968. *74* 91—95

Hamberger, L. A. and K. E. B Ahrén Effects of gonadotrophins in vitro on glucose uptake and lactic acid production of ovaries from prepubertal and hypophysectomized rats. *Endocrinology* 1967a. *81* 93—100.

Hamberger, L. and K. Ahrén Action of gonadotrophins on respiratory enzyme activity of isolated ovarian cells from the rat. *Acta endocr* (Kbh.) 1967 b. *113*. Suppl. 119 113.

Hamberger, L., J Sjöstrand and K. Ahrén, 1968 To be published.

HARRISON, R. J. The structure of the ovary I. *The Ovary I*. Ed. Zuckerman S. Academic Press New York and London 1962 pp. 143—187

HJALMARSON, A. and K. AHRÉN, Sensitivity of the rat diaphragm to growth hormone. *Acta endocr.* (Kbh.) 1967 *54* 645—662.

KERÉNYI G. and Z. PÓRSZÁSZ. In Mikrotechnische und histochemische Untersuchungsmethoden. *Akadémiai Kiadó*, Budapest 1964 pp. 576—579

KREBS, H. A., Enzyme activity and cellular structure. In *Horizons in biochemistry*. Eds. Kasha M. and B. Pullman. Academic Press London 1962, pp. 285—292.

KUN, E. and L. G. ABOOD, Colorimetric estimation of succinic dehydrogenase by triphenyl tetrazolium chloride. *Science* 1949 *109* 144—146

LINDSTRÖM, B., Roentgen absorption spectrophotometry in quantitative cytochemistry. *Acta radiol.* (Stockh.) 1955 Suppl. 125 1—197

LOSTROH A. J. and R. E. JOHNSON, Amounts of interstitial cell-stimulating hormone and follicle-stimulating hormone required for follicular development, uterine growth and ovulation in the hypophysectomized rat. *Endocrinology* 1966. *79* 991—996

LOWRY O. H., A quartz fibre balance. *J. biol. Chem.* 1941 *140* 183—189

LYON R. A., M. E. SIMPSON and H. M. EVANS, Qualitative changes in urinary gonadotrophins in human pregnancy during the period of rapid increase in hormonal titer. *Endocrinology* 1953. *53* 674—689

MANDL, A. M. and S. ZUCKERMAN. The growth of the oocyte and follicle in the adult rat. *J. Endocr.* 1952. *8* 126—132.

MEYER, R. K., W. H. MCSHAN and W. F. ERWAY. The succinic dehydrogenase activity of ovarian and lutein tissue. *Endocrinology* 1945. *37* 431—436.

MEYER, R. K., S. W. SOUKUP, W. H. MCSHAN and C. BIDDULPH, Succinic dehydrogenase in rat ovarian tissues during pregnancy and lactation. *Endocrinology* 1947 *41* 35—44

MOORE, W. W., Changes in pituitary LH concentration in prepubertal and postpubertal rats. *Neuroendocrinology* 1965/66. *1* 330—340.

POTTER, V. R., The homogenate technique. I. *Manometric techniques*. Eds. Umbreit, W. W., R. H. Burris and J. F. Stauffer. Burgess, Minneapolis 1957 pp. 170—187

PRICE, D. and E. ORTIZ, The relation of age to reactivity in the reproductive system of the rat. *Endocrinology* 1944 *34* 215—239

RAMIREZ, D. V. and S. M. MCCANN, Comparison of the regulation of luteinizing hormone (LH) secretion in immature and adult rats. *Endocrinology* 1963 *72* 452—464

RENNELS, E. G. Some factors influencing the cholesterol content of the interstitial tissue of the immature rat ovary. *Anat. Rec.* 1949. *103*. 520.

RENNELS, E. G., The influence of hormones on the histochemistry of ovarian interstitial tissue in the immature rat. *Amer. J. Anat.* 1951 *88* 63—108.

ROSENGREN, B. H. O. Determination of cell mass by direct X-ray absorption. *Acta radiol.* (Stockh.) 1959 Suppl. 178. 1—62.

RUBINSTEIN, L. Autoradiographic localization of glycine-³H incorporation in the isolated rat ovary. *Endocrinology* 1967 *80* 768—772.

RYAN, K. J. and R. V. SHORT. Formation of estradiol by granulosa and theca cells of the equine ovarian follicle. *Endocrinology* 1965. *76* 108—114

RYAN, K. J. and R. V. SHORT. Cholesterol formation by granulosa and theca cells of the equine follicle. *Endocrinology* 1966. *78* 214—216.

SELYE, H., J. B. COLLIP and D. L. THOMSON, On the effect of the anterior pituitary like hormone on the ovary of the hypophysectomized rat. *Endocrinology* 1933 *17* 494—500

SHINO M. and E. G. RENNELS, Pituitary follicle stimulating hormone [illegible] and ovarian development in the infant rat. *Endocrinology* 1967 *81* 1379—1386.

SLATER, E. C. The measurement of the cytochrome oxidase activity of enzyme preparations. *Biochem. J.* 1949 *44* 305—318

SOPER, E. H. and A. J. LADMAN, Responses to gonadotrophin in intact and hypophysectomized immature female rats as related to age. *Proc. Soc. exp. Biol.* (N.Y.) 1962 *111* 575—576

WESTMAN, A. Experimentelle Studien über die funktionelle Bedeutung der Theca-interna-Zellen. *Acta obstet. gyn. scand.* 1929 *8*. 290—306

WESTMAN, A., Untersuchungen über die Abhängigkeit der Funktion des Corpus Luteum von den Ovarialfollikeln und über die Bildungsstätte der Hormone im Ovarium. *Arch. Gynäk.* 1934 *158* 476—504

ZAJICEK, J. and E. ZEUTHEN. Quantitative determination by a special ampulla-diver of cholinesterase activity in individual cells, with notes on other uses of the method. I. *General Cytochem. Method. 2* Ed. Danielli, J. F. Academic Press, New York, 1961 pp. 131—152.

ZARROW M. X. and E. D. WILSON. The influence of age on superovulation in the immature rat and mouse. *Endocrinology* 1961 *69* 851—855.

ZEUTHEN E. Growth as related to the cell cycle in single-cell cultures of Tetrahymena pyriformis. *J. Embryol. exp. Morph.* 1953 *1* 239—249

Acta physiol. scand. 1958. 74. 426—439

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5-Hydroxytryptamine Release from Enterochromaffin Granules in vitro

By

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Received [illegible]

Abstract

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et al 1962) and recently Lembeck and Held (1966) found resemblances *in vitro* between release of 5-HT and norepinephrine.

Since little is known about enterochromaffin granules the influence of various factors on the release of 5-HT from enterochromaffin granules has been studied.

Material and methods

Material. A total of 132 cows and 115 pigs was studied. The cows were killed by shooting through the head and the pigs by electrocution. The material was obtained t the slaughterhouse within 10 to 15 mm after bleeding the animals. About 50 cm of the duodenum just caudal to the pyloric ring was removed and cooled in a sucrose solution containing many pieces of frozen sucrose solution and transported to the laboratory. All the preparative procedures were performed t 0° C and the sucrose solution used was isotonic (0 3 M, pH 6.9) unless otherwise stated. The duodenal mucous membrane was removed by scraping with glass slide and homogenized with eflon pestle homogenizer t 900 rpm. for 25 sec in tenfold olume of sucrose solution.

Preparation of the granule fraction. Large particles were removed by centrifugation in the cold t 800 × g for 10 min. The debris was suspended in a fivefold volume of sucrose solution and recentrifuged. The two supernatants were combined and centrifuged t 20,000 × g for 20 min. The loose top layer was removed from the pellet by swirling lightly and the bottom layer the so called "granule fraction was washed twice with sucrose solution. Finally the pellet was suspended in sucrose so that the protein concentration was 0.5 to 1.5 In the complete fractionation of the mucous membrane the heavy and light microsomes were separated at 50,000 × g for 50 min and t 105,000 × g for 60 min, respectively

Release experiments. For release studies 0.5 or 1 0 ml of the granule suspension nd 9 0 or 9.5 ml of the sucrose solution, phosphate buffer (0 15 M, pH 6.9) or phosphate buffer solution (0.15 M pH 6.9) containing the dissolved drug were included in plastic centrifuge tube. All the concentrations given are final concentrations in the incubation tubes. The incubations were carried out t 0 C—30° C for 30 or 60 min in water bath. Thereafter 10 ml of ice cold sucrose solution was added and the tubes were centrifuged t 35,000 × g for 10 min t 0 C. The pellet was washed once with cold sucrose solution, recentrifuged and finally suspended carefully in 7 ml of sucrose solution, frozen and preserved t —18° C for 5-HT determination. Controls, including sample kept 0 C (zero value) and sample incubated in the standard incubation medium, were treated as bove. The latter also was used as basis for estimating the 5-HT elease from the granule fraction. The spontaneous amine release the difference between the zero value and that obtained in the pure incubation medium—was alw ys determined. The results are given as percentage, with + when the amine release was retarded and—when it was ccelerated. The results are given as means ± S D. and the significance was estimated using Studen -test.

5-Hydroxytryptamine was determined using the spectrophotofluorometric method of Weissbach (1961) Of the chemicals used only reserpine interfered with the 5-HT determination and t was removed by chloroform extraction according to Lembeck and Held (1966)

Monoamine oxidase (MAO) activity was determined according to Lovenberg *et al* (1962)

Protein was determined by the method of Lowry *et al* (1951) Bovine serum albumin was used as standard.

For electron microscopic studies the pellet of the granule fraction was fixed in buffered glutaraldehyde solution (2.5 /v pH 7.2, 0° C for 2 hrs) nd the postfixation was carried out in 1 ( ) osmium etroxide solution, pH 7 4 (Rhodin 1954) A Philips EM 200 electron microscope was used.

Chemicals 1-adrenaline (Orion Oy) 1-noradrenaline (Orion Oy 1-α-(3 4-dihydroxyphenyl-)-lanine (Fluka AG), histamine dihydrochloride (E. Merck AG) indoleacetic cid (Fluka AG) creatinine sulphate salt of 5-hydroxytryptamine (Fluka AG ryptamine hydrochloride (Fluka AG), d,l-tryptophan (Fluka AG) tyramine h drochloride Sigma Chemical Comp corticotropin (N V Organon) cortisone acetate (Adreson N V Organon hydrocortisone acetate (Hydroadreson N V Organon) d,l-thyroxine (Sta Oy N-isonicot l-N benzylcarbamoyl) ethylhydrazine (Niamid Pfizer Co) N-isonicotinyl-N -isopropylh drazine Marsalid F Hoffmann-La Roche & Co AG) (N-3 phenylpropyl- 2 1 1-diphen lpropyl 3 -amine (Segontin R. Hoechst AG etylthiocholine iodide (Fluka AC -butyry) h h line iodide (Fluka AG) physostigmine (E Merck AG) hlorpromazine h drochl ride M & Baker Ltd) β-phe-)hisopropylhydrazine hydrochloride (Lakeside labora ories eserpi Serpasil Ciba AG) imipramine (Tofranil Geigy AG) mepyramine Orion h H k solution (Orion Oy histamine liberator compound 48 80 (Wellcome Research l ories pun (E. Merck AG guanethidine (Orion Oy triplex III and titriplex IV E Merck A

Other chemicals used were commercially ailable reagen grad p cts The concentrations of he hemicals in release experiments are given in the appropria b

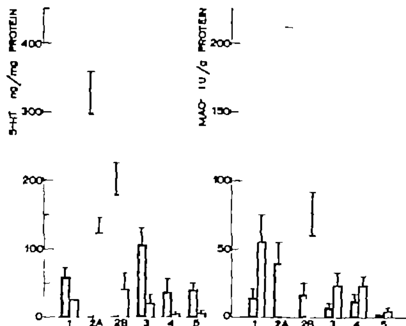


Fig. 1. 5-Hydroxytryptamine content and MAO activity in different fractions of the duodenal mucous membrane. 1: sediment obtained at 800 × g for 10 min; 2A: the compact bottom layer of the sediment obtained between 800 and 20,000 × g for 20 min; 2B: the loose top layer of the same fraction; 3: sediment obtained between 20,000 and 30,000 × g for 30 min; 4: sediment obtained between 30 000 and 105,000 × g for 60 min; 5: supernatant of the 4th fraction. For MAO activity pellets were suspended in 0.3 M sucrose solution, pH 6.9 and enzyme activity was measured at C for 60 min. Cow hatched, pig unhatched. Means and standard deviations.

Results

1. *Fractionation of the mucous membrane.* The results are presented in Fig. 1. The highest 5-HT content was in the fraction sedimented between 800 and 20 000 g. MAO activity was highest in the granule fraction.

2. *Purity of the granule fraction.* For electron microscopy an intact tissue containing strongly osmiophilic granules was used as a standard when estimating the morphology of the isolated granule fraction. As seen in Fig. 2, dense osmiophilic granules made up only a small part of the total organelle population in the granule fraction. These granules had a strong affinity for osmium tetroxide, a fine reticular inner structure, a smooth surrounding membrane and a size similar to or larger than that estimated *in situ* in the enterochromaffin cell cytoplasm. The granule fraction displayed several other organelles, *e.g.* intact and ruptured mitochondria and many types of vesicles with a low affinity for osmium.

3. *Effect of temperature.* The results are summarized in Table I. In both species increasing the temperature accelerated the 5-HT release significantly. Freezing and thawing released 5-HT almost entirely (Cow -72.9 ± 6.0 %, pig -67.8 ± 8.8 %, $n = 6$).

4. *Effect of molarity and pH.* In phosphate buffer and sucrose solutions the 5-HT release



Fig. 2. Electron micrograph of the bottom ... from cow duodenal mucous membrane ... particles re easily distinguishable among other ... and Methods 40,000

was proportional to the decrease in ... incubation medium (Table II and III) ... complet in 30 min.

3 *Effect f detergents* All the deterge... release from the granul fraction (Tabl...

6 *Effect of buffer and salt solutions* As seen ... the salt nd buffer solutions than in the ... granule fraction in a sucrose medium ... magnesium hloride retarded 5-HT ... acceleration n amine release The strong ... the denaturing effect of the metal on the ...

7 *Effect f hormones and enzymes* Only hyd... chemical were studied owing to their ... U I /ml) and thyroxin (2mM) had n...

TABLE I Effect of temperature on the release of 5-HT from the granule fraction. Incubation medium: 0.3 M sucrose solution pH 6.9. The 5-HT value of the sediment in 0.3 M sucrose solution incubated at 0 C served as basis. The mean zero value was 0.31 µg 5-HT/mg protein for cow and 0.0[illegible] for pig

Temperature	Species	n	Incubation time	Per cent 5-HT release mean	S. D.	p<
5 C	Cow	6	60 min	−3.1	4.9	
5 C		6	24 hrs	−55.8	17.5	0.001
23 C		6	30 min	−10.6	7.0	0.005
23 C		6	60 min	−21.4	7.7	0.001
37° C		6	30 min	−39.1	10.7	0.001
37 C		6	60 min	−42.1	10.3	0.001
50 C		6	30 min	−81.9	8.1	0.001
50° C	"	6	60 min	−92.6	8.0	0.001
5 C	Pig	6	60 min	−3.7	5.0	
C		6	24 hrs	−72.0	14.4	0.001
23 C		6	30 min	−21.7	5.8	0.001
23 C		6	60 min	−44.4	11.1	0.001
37 C		6	30 min	−48.8	10.3	0.001
37 C		6	60 min	−72.0	8.5	0.001
50 C		6	30 min	−92.7	2.0	0.001
50 C		6	60 min	96.1	1.4	0.001

TABLE II Effect of concentration of the sucrose solution on 5-HT release from the granule fraction. Incubations were carried out at 23 C for 30 min. Incubation media: sucrose solutions and distilled water pH of which was adjusted to 6.9 with 0.1 N NaOH or 0.1 HCl. 5-HT release is expressed as the percentage loss of the 5-HT content of the sediment as compared with the value obtained after incubation in 0.3 M sucrose solution, pH 6.9. The mean zero value was 0.39 µg 5-HT/mg protein for cow and 0.10 for pig. The spontaneous 5-HT release was 18.9 ± 5.2 for cow and 29.1 ± 8.4 for pig

Concentration M	Species	n	Per cent 5-HT release Mean	S. D.	p
0.15	Cow	6	43.5	6.2	0.001
0.15	Pig	6	−43.1	5.5	0.001
0.075	Cow	6	−85.6	5.8	0.001
0.07	Pig	6	−82.7	6.7	0.001
0.030	Cow	6	−93.6	2.9	0.001
0.030	Pig	6	92.5	4.0	0.001
Dist. water	Cow	6	99.5	0.26	0.001
Dist. water	Pig	6	−97.5	0.71	0.001

TABLE III Effect of concentration and pH of the phosphate buffer on 5-HT release from the granule fraction. Experimental conditions as in Table II, except for the incubation time of 60 min. The mean zero value was 0.23 μg 5-HT/mg protein for cow and 0.10 for pig. The spontaneous 5-HT release was + 24 4 ∓ 12.8 for cow and 40.9 ∓ 8.2 for pig

Phosphate Buffer/M	Species	n	pH	Per cent 5-HT release Mean	S. D.	p<
0.075	Cow	6	5.5	—60.0	8 6	0 001
0 075		6	6 9	—90.5	6.0	0.001
0 075	"	6	8.0	—97 6	2.6	0.001
0.100	"	6	5.5	—44.5	13.4	0.001
0.100	"	6	6.9	—55.5	11.9	0.001
0.100	"	6	8.0	—68.5	6.1	0 001
0 150		6	5.5	—21 7	10.2	0.0025
0 150		6	6.9	—47 1	8.5	0 001
0.150	"	6	8.9	—63 6	9.0	0.001
0 075	Pig	6	5.5	—60.7	11.0	0.001
0.075		6	6.9	—83.0	6.2	0.001
0 075		6	8.0	—97 4	3.1	0 001
0.100		6	5.5	—32.7	10.7	0.001
0.100		6	6.9	—72 0	17.8	0 001
0.100		6	8.0	—93.5	6.6	0.001
0 150		6	5 5	—25 7	12.6	0.0025
0.150		6	6.9	—41.9	22.0	0 0025
0.150		6	8 0	—81.9	9 7	0.001

(0.63 mg/ml, release —56 0 in cow and —41 4 in pig) and hydrocortison (0.63 mg/ml, release —21 6 in cow and —54 7 in pig) released 5-HT significantly ($p<0.05$—0 001) from the granule fraction of both species during 60 min at 23° C Trypsin (100 I U /ml release —78 0 in cow and —63 6 in pig nduced a highly significant ($p<0$ 001) 5-HT release from the granules during 60 min t room temperature

8 *Effect of amino acids and their derivatives* Adrenaline (2mM) noradrenalin 2mM dihydroxyphenylalanin (2mM) 3-hydroxytyramine (4mM) tryptophan (2mM) and indolylacetic acid (2mM) had no significant effect on amine release from the granule fraction. Only tyramin (4mM, release —68 4 in cow nd —53 8 in pig) caused a significant ($p<0$ 001) 5-HT release during 30 min a room temperature. In a tenfold dilution tyramine had no effect. In both spec es histamine (2mM release —14.2 in cow and —6 7 in pig) accelerated amin release slightly ($p<0.01$—0 02)

TABLE IX Effect of detergents on 5-HT release from the granule fraction. Experimental conditions as in Table II except that the incubations were carried out in 0.15 M phosphate buffer pH 6.9 and with sample in pure buffer as reference. The mean zero value was 0.16 µg 5-HT/mg protein for cow and 0.10 for pig. The spontaneous 5-HT release was + 27.1 ± 4.3 for cow and + 22.2 ± 7.6 for pig

Detergent	Concentration		Species	n	Per cent 5-HT release		p
					Mean	S.D.	
Sodium desoxycholate	0.01 M		Cow	5	−91.5	3.1	0.001
	0.01 M		Pig	5	−97.7	2.1	0.001
Sodium taurocholate	0.01 M		Cow	5	−92.9	4.1	0.001
	0.01 M		Pig	5	−87.2	10.9	0.001
Saponin	0.4	(w/v)	Cow	5	−87.5	1.1	0.001
"	0.4	(w/v)	Pig	5	−98.5	1.6	0.001
Triton® X—100	0.4	(v/v)	Cow	5	−99.1	1.2	0.001
	0.4	(v/v)	Pig	5	−98.5	1.8	0.001

9. *Effect of MAO inhibitors.* MAO inhibitors were the only chemicals tested that retarded 5-HT release in a 0.15 M phosphate buffer solution. However the ability of these inhibitors to retard amine release was clearly weaker than that of the isotonic sucrose solution (Table VII).

10. *Effect of pharmacological drugs.* Acetylcholine chloride (1mM) or butyrylcholine (1mM) with eserine (1mM) did *not* release 5-HT from the granules during 30 min at room temperature. Guanethidine (1mM–0.01 mM) was also inactive. The effects of some other drugs on 5-HT release are shown in Table VIII.

Discussion

The enterochromaffin system. Enterochromaffin cells make up only a small proportion of the epithelial cell population in the duodenal mucous membrane of mammals. Therefore it was not surprising that the granule fraction used in the present experiments showed only a small content of osmiophilic granules. In preliminary studies, the highest 5-HT content (µg/mg protein) was found in the heaviest fraction of the gradient (0.8 to 2.0 M sucrose gradient) centrifugation. Since the osmiophilic granules in this preparation were accompanied by large amounts of membrane fragments density gradient centrifugation was no better than the simpler technique finally adapted. The osmiophilic granules seen in electron micrographs may not all be derived from enterochromaffin cells. The granules of dopamine cells, for instance, look much the same but have a larger diameter in the intact tissue (Penttilä 1967 unpubl.). There are considerable morphological differences between the fine structure of the enterochromaffin (Wetzstein and Doerfler 1962) and adrenergic (Potter 1966) granules. This indicates a basic dissimilarity between these types of granules.

TABLE V. 5-HT release from the granule fraction incubated in various buffer and salt solutions. Experimental conditions as in Table II. The mean zero value in the phosphate, tris HCl and glycylglycine experiments was 0.37 μg 5-HT/mg protein for cow and 0.13 for pig. The spontaneous 5-HT release was $+15.0 \mp 8.5$ % for cow and $+21.1 \mp 7.3$ for pig. In the other experiments the corresponding mean values were 0.19 and 0.06 μg 5-HT/mg protein, respectively and the spontaneous release $+25.0 \mp 5.8$ % and $+44.2 \mp 16.5$ % respectively.

Solution	*Concentration*	*Species*	*n*	*Per cent 5-HT release* Mean	S. D.	$p<$
Phosphate	0.15 M	Cow	6	−19.5	6.9	0.001
	0.15 M	Pig	6	−17.7	6.3	0.001
Tris-HCl	0.3 M	Cow	6	−20.2	4.5	0.001
	0.3 M	Pig	6	−19.7	5.9	0.001
Glycylglycine	0.3 M	Cow	6	−31.7	11.3	0.001
	0.3 M	Pig	6	−25.4	7.6	0.001
$CaCl_2$	0.6 % (w/v)	Cow	5	−70.8	10.7	0.001
	0.6 % (w/v)	Pig	5	−71.8	12.5	0.001
KCl	0.9 % (w/v)	Cow	5	−55.0	10.5	0.001
	0.9 % (w/v)	Pig	5	−61.6	9.0	0.001
$MgCl_2$	0.6 % (w/v)	Cow	5	−58.9	6.6	0.001
	0.6 % (w/v)	Pig	5	−63.9	7.5	0.001
NaCl	0.9 % (w/v)	Cow	5	−50.9	15.2	0.001
	0.9 % (w/v)	Pig	5	−58.9	9.8	0.001
Hanks solution		Cow	6	−38.5	20.9	0.001
		Pig	5	−68.7	9.1	0.001
Krebs-Ringer solution		Cow	6	−48.6	26.3	0.001
		Pig	5	−72.0	13.3	0.001
Tyrode's solution		Cow	6	−66.3	14.5	0.001
		Pig	5	−74.6	11.5	0.001

The similar shape, size distribution, fluorescence properties and silver staining reactions of the enterochromaffin system in different mammals (Penttila 1966) indicate that it is composed of one principal cell type. This similarity between various species is supported by the identical features of the amine release mechanism from pig and cow granules observed in the present study.

Amine release in various systems. It is known that 5-HT is liberated from enterochromaffin cells in vivo by reserpine (Benditt and Wong 1957) and by intestinal motility and pressure (Bülbring and Crema 1959, Kärki *et al.* 1960). In this study most of the

TABLE VI Effect of some cations and chelating agents on 5-HT release from the granule fraction. Experimental conditions as in Table III. The chemicals were dissolved in 0.3 M sucrose solution, and the pH was adjusted to 6.9. The mean zero value was 0.?9 µg 5-HT/mg protein for cow and 0.11 for pig. The spontaneous 5-HT release was + 24.3 ∓ 7.0 for cow and + 38.1 ∓ 9.1 for pig

Chemical	Concentration/mM	Species	n	Per cent 5-HT release Mean	S. D	p<
$CaCl_2$	4	Cow	8	−11.1	16.1	0.05
"	4	Pig	5	−15.4	11.6	0.025
$MgCl_2$	4	Cow	9	+19.3	22.2	0.025
"	4	Pig	5	+ 8.2	7.7	0.05
$CuCl_2$	2	Cow	5	−92.1	4.6	0.001
"	2	Pig	5	−88.8	10.5	0.001
Titriplex III®	20	Cow	9	−16.4	15.1	0.005
	20	Pig	5	− 6.3	4.5	0.025
Titriplex IV®	10	Cow	6	−14.0	8.1	0.005
"	10	Pig	6	−10.8	6.1	0.005

TABLE VII Effect of MAO inhibitors on 5-HT release from the granule fraction. Experimental conditions as in Table IV with an incubation time of 60 min. The mean zero value was 0.35 µg 5-HT/mg protein for cow and 0.11 for pig

Drug	Concentration/mM	Species	n	Per cent 5-HT release Mean	S. D	p<
Catron®	2	Cow	5	+21.1	16.1	0.025
"	2	Pig	5	+32.8	10.9	0.0025
Marsilid®	4	Cow	5	−33.9	10.8	0.001
	4	Pig	5	−35.5	9.1	0.001
Nialamide	4	Cow	5	+27.9	22.0	0.025
"	4	Pig	5	+30.2	10.9	0.0025
Sucrose	300	Cow	5	−50.3	13.1	0.001
"	300	Pig	5	−45.8	14.1	0.001

pharmacological agents tested were effective *in vitro* only in unphysiologically high concentrations. It is therefore difficult to draw any conclusions about the effect of the agents *in vivo*.

A study of the effects of varying incubation conditions and addition of various drugs on the release of amines indicates many similarities between a number of

TABLE VIII Effect of various drugs on 5-HT release from the granule fraction. Experimental conditions as in Table VII. The mean zero value was 0.37 µg 5-HT/mg protein for cow and 0.09 for pig. The spontaneous 5-HT release was + 40.1 ∓ 8.1 % for cow and and + 45.1 ∓ 6.5 for pig

Drug	Concentration/mM	Species		Per cent 5-HT release Mean	S.D.	p<
Chlorpromazine	2.0	Cow	6	−86.4	4.7	0.001
"	0.2		6	−48.4	16.8	0.001
	0.02	"	6	−13.3	9.1	0.01
	2.0	Pig	5	−92.0	9.6	0.001
	0.2	"	6	−57.1	19.0	0.001
	0.02	"	6	−10.0	8.0	0.025
Imipramine	1.0	Cow	6	−89.9	8.1	0.001
	0.1		6	−43.8	19.1	0.0025
	0.01	"	6	−13.5	10.0	0.025
	1.0	Pig	5	−79.9	11.8	0.001
	0.1		5	−31.1	10.0	0.001
	0.01	"	5	− 8.1	6.1	0.025
Mepyramine	1.0	Cow	5	−55.0	17.1	0.001
	0.01		5	+ 1.2	4.9	
	1.0	Pig	5	−41.1	16.1	0.001
	0.01		5	− 1.9	5.4	
Reserpine	1.0	Cow	5	+ 5.4	4.2	0.025
	1.0	Pig	5	+14.6	5.7	0.0025
Segontin®	2.0	Cow	5	−89.6	3.6	0.001
	0.2		5	−21.9	11.1	0.005
	0.02		5	−10.8	5.0	0.0025
	2.0	Pig	5	−93.4	4.4	0.001
	0.2		5	−57.1	19.0	0.001
	0.02		5	−10.1	8.1	0.025

systems. There are the release of 5-HT from enterochromaffin granules, the release of norepinephrine from adrenal medullary (Hillarp 1958, Euler and Lishajko 1961 a and b, Schümann and Philippu 1961, 1963, Euler *et al.* 1964, Stjärne 1964, Stjärne and Lishajko 1966) or from adrenergic granules (Euler and Lishajko 1961 a and b, Euler *et al.* 1963, Stjärne 1964, 1966, Potter 1966), the release of 5-HT from mast cells (Carchman-Arocha 1961, 1962, McLean *et al.* 1962, Penttila and Jansson 1967) and from platelets (Stacey 1958, Gurman and Schanberg 1962, Schöne and Lindner 1960, McLean *et al.* 1963, Paasonen 1965).

There are some differences between the release of 5-HT from enterochromaffin

granules and the release of amines from other depots 1) Temperature had a stronger effect on enterochromaffin granules than on mast cells (Penttilä and Jansson 1967) or neural granules (Euler and Lishajko 1961 a) 2) Neural granules are more resistant to freezing and thawing (Euler and Lishajko 1961 a) 3) Enterochromaffin granules loose their 5-HT during incubation in isotonic salt solutions more easily than do the adrenal medullary (Hillarp 1958) and adrenergic granules (Euler and Lishajko 1961 a, Potter and Axelrod 1963 Potter 1966) 4) Adrenergic granules are also more resistant in hypotonic solutions (Potter 1966) and 5) withstand detergents better (Potter and Axelrod 1963) 6) In contrast to adrenergic granules (Euler and Lishajko 1961 a, Potter and Axelrod 1963) and mast cells (Jansson and Penttilä 1967 unpubl.) 5-HT release from enterochromaffin granules was accelerated at pH 5—8. 7) Histamine liberator compound 48/80 liberates 5-HT from mast cells both *in vivo* and *in vitro* (Carchia Arocha 1961 Schievelbein and Zitzelsberger 1964) but has no effect upon enterochromaffin granules. 8) Chlorpromazine had a strong effect upon enterochromaffin granules but not on adrenergic granules at or below 10^{-4}M (Potter 1966) 9 Reserpine is a potent amine releaser *in vitro* in other system but had no effect in this study

It is evident that some of the differences discussed may have resulted from the differences in the experimental techniques and the material (intact cells, isolated organelles as well as the amine used by the various authors.

Possible binding mechanism of 5-HT in enterochromaffin granules There are no satisfactory data on the chemical composition of the enterochromaffin granules, since no homogenous preparations have been available. Prusoff (1960) has suggested that ATP is concentrated in these granules and may be involved in 5-HT binding as suggested for adrenal medullary granules by Hillarp *et al.* (1955) However Prusoff gave no morphological data on the purity of his granule fraction. At physiological pH the amine group of 5-HT is almost completely protonated (Vane 1959 whereas ATP is negatively charged see Burard 1964 Therefore an ionic type of binding seems obvious This does not exclude other types of binding such as one involving the chemically-uncharacterized protein moiety

In contrast to intact mast cells Jansson and Penttilä 1967) enterochromaffin granules easily released 5-HT in various isotonic salt solutions This indicates that 5-HT is bound by weak forces in the granule matrix. Similar observations have been made for histamine in isolated mast cell granules Åborg *et al* 1967 On the other hand at various pH 5-HT was liberated from enterochromaffin granules in the present study in quite a different way to liberation from intact mast cells (Jansson and Penttilä 1967 and isolated mast cell granules Åborg *et al* 1967) This shows that the binding mechanism of 5-HT by enterochromaffin and by mast cells may be basically different. The two types of granule are not directly comparable because mast cells contain heparin and zinc ions (Green 1966 and have a granular fine structure unlike that of enterochromaffin granules Lagunoff 1966)

In the present study chelating agents were able to liberate 5-HT to a minor extent. Possibly their action involves the divalent ions that can interact in enterochromaffin

granules as earlier suggested for amine binding and storage in mast cells (Green 1966) and for accumulation of norepinephrine in adrenal medullary granules (Carlsson *et al.* 1965) On the other hand the Ca-induced and Mg retarded 5-HT release from enterochromaffin granules was of a similar order to that observed earlier by Lembeck and Held (1966)

Possible release mechanisms There are no earlier reports on the accumulation of biogenic amines by isolated enterochromaffin granules. Only tyramine of the amines tested in the present study was able to liberate 5-HT possibly by replacing it in the granules, as suggested by Schümann and Philippu (1963) for adrenal medullary granules. The other amines tested were without effect and it remains to be determined whether various amines accumulate in enterochromaffin granules *in vitro* In fluorescence microscopy intact mast cells (Jansson and Penttilä 1967) took up dopamine, but 5-HT was not liberated. This suggests that there may be several types of binding site for biogenic amines in the granular matrix of mast cells.

In the present study monoamine oxidase inhibitors were the only agents that caused a significant retardation of 5-HT release. The fraction collected between 800 and 20 000 *g* contained a high monoamine oxidase activity possibly owing to the presence of mitochondria, which have been reported to contain about 3/4 of the cell s enzyme activity (Pletscher *et al.* 1966) The enzymatic degradation of 5-HT may be prevented by inhibition of monoamine oxidase activity whereby the concentration of 5-HT in solution is maintained. This would then counteract release of the amine and favour its reaccumulation in granules.

Segontin® and reserpine at higher concentrations *in vitro* deplete various amine depots in a similar way (Paasonen 1965 Euler *et al* 1964) It is obvious that the lack of effect of reserpine in the present study may be due to the exogenous conditions used, which could nullify the mechanism suggested for 5-HT release by platelets (Paasonen 1965)

It is obvious that the granular matrix structure is important for amine-binding *In vitro* this structure could easily be broken and amine release may occur then secondarily The denaturing agents and heavy metal studied are strong disruptors of protein configuration and were also very potent amine releasers. Obviously the 5-HT depleting effect of trypsin, as earlier observed for chymotrypsin (Lembeck and Held 1966) was also based on damaging the granular membrane or protein matrix.

There are no earlier reports on the effect of cortisone and hydrocortisone on amine release *in vitro* In the present study relatively high concentration of these hormones significantly accelerated amine release from the granul fra tion *In vivo* cortisone has been reported to cause a slight depression of the 5-HT ontent of platelets and rat intestine (Cass and Marshall 1962) but an increase in enterochromaffin cell number and 5-HT content i the duodenum of the newborn rat Penttilä 1966)

References

ÅBORG, C. H., J. NOVOTNY and B. UVNÄS, Ionic binding of histamine in mast cell granules. *Acta physiol. scand.* 1967. 69. 276—283.

BENDITT, E. P. and R. L. WONG, On the concentration of 5-hydroxytryptamine in mammalian enterochromaffin cells and its release by reserpine. *J. exp. Med.* 1957. 105. 509—520.

BLASCHKO, H. and A. D. WELCH, Localization of adrenaline in cytoplasmic particles of the bovine adrenal medulla. *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1953. 219. 17—22.

BULBRING, E. and A. CREMA, The release of 5-hydroxytryptamine in relation to pressure exerted on the intestinal mucosa. *J. Physiol. (Lond.)* 1959. 146. 18—28.

BUZARD, J. A., In: *Actions of hormones on molecular processes.* Ed. G. Litwack and D. Kritchevsky. Wiley, New York, London, Sydney. 1964. 320—335.

CARCHMAN-ARBOGA, H., Release of histamine and 5-hydroxytryptamine from cells of the peritoneal fluid of rats. *Canad. J. Biochem.* 1961. 39. 395—402.

CARLSSON, A., N.-Å. HILLARP and B. WALDECK, Analysis of the Mg^{++}—ATP dependent storage mechanism in the amine granules of the adrenal medulla. *Acta physiol. scand.* 1963. 59. Suppl. 215.

CASS, R. and P. B. MARSHALL, Effect of adrenocortical hormones on tissue histamine and 5-hydroxytryptamine in the rat. *Arch. int. Pharmacodyn.* 1962. 136. 311—332.

EULER, U. S. v. and N.-Å. HILLARP, Evidence for the presence of noradrenaline in submicroscopic structures of adrenergic axons. *Nature* (Lond.) 1956. 177. 44—45.

EULER, U. S. v. and F. LISHAJKO, Noradrenaline release from isolated nerve granules. *Acta physiol. scand.* 1961a. 51. 193—203.

EULER, U. S. v. and F. LISHAJKO, Effect of reserpine on the release of catecholamines from isolated nerve and chromaffine granules. *Acta physiol. scand.* 1961b. 52. 137—145.

EULER, U. S. v., L. STJÄRNE and F. LISHAJKO, Catecholamines and adenosine triphosphate in isolated adrenergic nerve granules. *Acta physiol. scand.* 1963. 59. 495—496.

EULER, U. S. v., L. STJÄRNE and F. LISHAJKO, Effects of reserpine, Segontin and phenoxybenzamine on the catecholamines and ATP of isolated nerve and adrenomedullary storage granules. *Life Sci.* 1964. 3. 35—40.

GIARMAN, N. J. and S. M. SCHANBERG, Drug-induced alterations of the sub-cellular distribution of 5-hydroxytryptamine (serotonin). *Biochem. Pharmacol.* 1962. 9. 93—96.

GREEN, J. P., Synthesis, uptake and binding of histamine and 5-hydroxytryptamine in mast cells. In: *Mechanisms of Release of Biogenic Amines.* Ed. U. S. VON EULER, S. ROSELL and B. UVNÄS. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Paris, Braunschweig. 1966. 125—145.

HILLARP, N.-Å., The release of catecholamines from the amine containing granules of the adrenal medulla. *Acta physiol. scand.* 1958. 43. 292—302.

HILLARP, N.-Å., B. HÖGBERG and B. NILSON, Adenosine triphosphate in the adrenal medulla of the cow. *Nature* (Lond.) 1955. 176. 1032—1033.

KÄRKI, N. T., M. K. PAASONEN and P. PELTOLA, Factors influencing the release of 5-hydroxytryptamine from the rat brain and intestine by rauwolfia alkaloids. *Ann. Med. exp. Fenn.* 1960. 38. 231—236.

LAGUNOFF, D., Structural aspects of histamine binding. The mast cell granule. In: *Mechanisms of Release of Biogenic Amines.* Ed. U. S. VON EULER, S. ROSELL and B. UVNÄS. Pergamon Press, Oxford, London, Edinburgh, New York, Toronto, Paris, Braunschweig. 79—94. 1966.

LEMBECK, F. and H. HELD, Serotoninfreisetzung aus isolierten Granula enterochromaffiner Zellen. *Naunyn-Schmiedeberg Arch. exp. Path. Pharmak.* 1966. 253. 409—420.

LOVENBERG, W., R. J. LEVINE and A. SJOERDSMA, A sensitive assay of monoamine oxidase activity in vitro: application to heart and sympathetic ganglia. *J. Pharmacol. exp. Ther.* 1962. 135. 7—10.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenolreagent. *J. biol. Chem.* 1951. 193. 265—275.

MCLEAN, J. A., NICHOLSON and D. HERTLER, Amine transport and storage in rabbit platelets. *Life Sci.* 1963. 9. 683—690.

MORAN, N. C., B. UVNÄS and B. WESTERHOLM, Release of 5-hydroxytryptamine and histamine from rat mast cells. *Acta physiol. scand.* 1962. 56. 26—41.

PAASONEN, M., Release of 5-hydroxytryptamine from blood platelets. *J. Pharm. Pharmacol.* 1965. 17. 681—697.

PENTTILÄ, A., Histochemical reactions of the enterochromaffin cells and the 5-hydroxytryptamine content of the mammalian duodenum. *Acta physiol. scand.* 1966. 69. Suppl. 281. 1—77.

PENTTILÄ, A. and S. E. JANSSON, 5-Hydroxytryptamine release and associated morphological changes of mast cells in vitro. *Exp. Cell Res.* 1967. In Press.

PLETSCHER, A., K. F. GEY and W. P. BURKARD, Inhibitors of monoamine oxidase and decarboxylase of aromatic amino acids. In: *Handb. d. exp. Pharmakol.* Ed. V. Erspamer. Springer Verlag Berlin, Heidelberg, New York. 1966. 593—735.

POTTER, L. T. Storage of norepinephrine in sympathetic nerves. *Pharmacol. Rev.* 1966, *18*, 439—451.

POTTER, L. T., and J. AXELROD, Properties of norepinephrine storage particles of the rat heart. *J. Pharmacol.* 1963, *142*, 299—305.

PRUSOFF, W. H., The distribution of 5-hydroxytryptamine and adenosinetriphosphate in cytoplasmic particles of the dog's small intestine. *Brit. J. Pharmacol.* 1960, *15*, 520—524.

RHODIN, J. Thesis. Correlation of ultrastructural organization and function in normal and experimentally changed proximal tubule cells of the mouse kidney. *Aktieb. Godvil. Stockholm.* 1954. 1—76.

SCHIEVELBEIN, H., and B. ZITZELSBERGER, Vergleichende Untersuchungen zum Transport von Histamin, 5-Hydroxytryptamin und Catecholaminen. I. Freisetzung von biogenen Aminen. *Med. exp.* 1964, *11*, 239—246.

SCHÖNE, H. H., and E. LINDNER, Die Wirkung des N-(3'-Phenyl-propyl-(2'))-1,1-diphenyl-propyl-(3)-amins auf den Stoffwechsel von Serotonin und Noradrenalin. *Arzneimitt.-Forsch.* 1960, *10*, 583—585.

SCHÜMANN, H. J., and A. PHILIPPU, Untersuchungen zum Mechanismus der Freisetzung von Brenzcatechinaminen durch Tyramin. *Arch. exp. Path. Pharmak.* 1961, *241*, 273—280.

SCHÜMANN, H. J., and A. PHILIPPU, Zum Mechanismus der durch Calcium und Magnesium verursachten Freisetzung der Nebennierenmark-Hormone. *Arch. exp. Path. Pharmak.* 1963, *244*, 466—476.

STACEY, R. S. In *5-Hydroxytryptamine*. Ed. G. P. LEWIS, London: Pergamon Press 1958. 116—122.

STJÄRNE, L., Studies of catecholamine uptake storage and release mechanisms. *Acta physiol. scand.* 1964, *62*, Suppl. 228.

STJÄRNE, L. Storage particles in noradrenergic tissues. *Pharmacol. Rev.* 1966, *18*, 425—432.

STJÄRNE, L., and F. LISHAJKO, Comparison of spontaneous loss of catecholamines and ATP *in vitro* from isolated bovine adrenomedullary, vesicular gland, vas deferens and splenic nerve granules. *J. Neurochem.* 1966, *13*, 1213—1216.

VANE, J. R., The relative activities of some tryptamine analogues on the isolated rat stomach strip preparation. *Brit. J. Pharmacol.* 1959, *14*, 87—98.

WEISSBACH, H., The estimation of serotonin in biological material. *Stand. Meth. Clin. Chem.* 1961, *4*, 197—203.

WETZSTEIN, R., and W. DOERFLER, Elektronenmikroskopie enterochromaffiner Zellen. *Anat. Anz. Erg. Heft* 1962, *111*, 113—120.

WETZSTEIN, R., W. DOERFLER and S. SCHWINK, Die Feinstruktur der enterochromaffin Zellen und ihrer spezifischen Granula. Elektronenmikroskopische Untersuchungen am Duodenum des Meerschweinchens. *Protoplasma* (Wien) 1962, *55*, 303—312.

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Proximal Luminal Volume and Fluid Reabsorption in the Rat Kidney[1]

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Abstract

BAINES, A. D., C. W. GOTTSCHALK and P. P. LEYSSAC. *Proximal luminal volume and fluid reabsorption in the rat kidney.* Acta physiol. scand. 1968. *74.* 440—452.

The relationship between proximal luminal volume and proximal salt and water reabsorption was studied in 21 non-diuretic anesthetized male rats. Luminal diameters were measured in enlarged photographs of the surface of the capsulated kidney. Proximal reabsorption rates were estimated from Cin, which changed spontaneously in the range 0.4—1.6 ml/min g kidney weight (k.w.). Cin was further reduced in eight rats by compression of the renal artery. The mean proximal luminal diameter was 18.7 μ without compression of the renal artery and 19.6 μ during compression. There was no consistent relationship between estimated proximal reabsorption rate and proximal luminal diameter. It is concluded that proximal tubular volume was not the prime determinant of proximal salt and water reabsorption rate in rats under the conditions of these experiments.

A conflict between the implications of a hypothesis and the results of a technique prompted this investigation of the relationship between luminal volume of the proximal tubules and glomerular filtration rate (GFR). The hypothesis states that intraluminal volume is a determinant of proximal tubular salt and water reabsorption rate (Gertz *et al.* 1965, Rector *et al.* 1966, Brunner *et al.* 1966, Rector *et al.* 1967). In the technique the time it takes the lumen of superficial proximal convolutions to disappear after clamping the aorta or renal artery—the occlusion time—is used as a measure of proximal salt and water reabsorption rate (Leyssac 1963, Leyssac 1964 a).

The conflict arises when hypothesis and technique are applied to the problem of the proportionality between GFR and proximal tubular reabsorption. There is a

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proportionality between GFR and overall tubular reabsorption in nondiuretic rats since changes in GFR are much greater than changes in urine flow Leyssac found that "glomerulo-tubular balance" in nondiuretic rats was due primarily to parallel fluctuations in GFR and *proximal* tubular reabsorption rate (Leyssac 1963) as has been generally accepted (Gottschalk 1963 Giebisch and Windhager 1964 Glabman *et al.* 1965) His studies were done using the occlusion time technique on the assumption that proximal tubular volume did not vary with inulin clearance (Cin) and therefore the volume reabsorbed after abrupt interruption of renal blood flow was constant. However Rector and his co-workers (1966 1967) have concluded from their experiments that tubular geometry is of critical importance in the maintenance of constant fractional reabsorption in the proximal tubule. They suggested that, under a given physiological condition, glomerular-tubular balance is maintained because tubular volume and GFR decrease together If this theory applies to nondiuretic rats, the occlusion time (OT) should remain constant or become shorter as GFR decreases and the volume to be reabsorbed is reduced. Instead Leyssac found that the OT became longer as GFR decreased, suggesting that factors other than luminal volume were responsible for the maintenance of relatively constant fractional reabsorption in the proximal tubules of nondiuretic rats (1963 1964 a)

The present study was undertaken to determine whether or not proximal tubular volume changed in direct proportion to variations in proximal reabsorption rate under nondiuretic conditions. Leyssac (1963) failed to find such a relationship when he measured luminal diameters in freeze-substituted sections from rapidly frozen kidneys however artifacts due to the removal of the kidney as well as the histological technique might have obscured the relationship predicted from the above hypothesis. Therefore in this study tubular volume was estimated by measuring proximal luminal diameters in microphotographs of the kidney surface in vivo Reabsorption rate was inferred from the inulin clearance. Luminal volume was found to be relatively constant and independent of Cin.

Methods

Twenty-one 220—270 g male Wistar rats, allowed free access to food and water were anesthetized with pentobarbital and/or amobarbital and prepared for clearance measurements with their left kidneys exposed as previously described (Leyssac 1963 Gottschalk and Mylle 1956) A short segment of the renal artery was dissected in eight rats. Cotton wool soaked in mineral oil was placed about the kidney which was bathed with oil. The exposed surface was covered with a cover slip supported by the surrounding cotton so that the kidney surface was not compressed. The kidney was not decapsulated but in eight experiments was freed from the posterior abdominal wall and a loop of thread placed loosely about the hilus At the end of the latter experiments an intravenous injection of ferrocyanide was given and the kidney frozen in situ. Ferrocyanide transit time was measured in these kidneys by the method of Hanssen (1958) and will be reported upon in a subsequent publication Rectal temperatures remained between 36 and 38 C throughout the experiments

A priming dose of 60 μc H3 inulin was given in 0 3 ml of saline and followed by a continuous infusion at 0.007 ml/min 1 4 μc/min At least forty and usually 60 min elapsed between the priming injection and the beginning of the first clearance period Each period began and ended with the collection of an arterial blood sample and included two to four serial 5—30 min urine collections. Urine was collected directly in calibrated polyethylene tubing (PE 50) and the volume excreted was determined from the length of the urine column. Radio-

activity in plasma and urine was measured in a modified Bray' solution (Lassiter and Gottschalk 1964) using a liquid scintillation spectrometer

To ascertain the constancy of flow and check on the accuracy of measurement 2 to 4 consecutive urine samples were collected in each clearance period the size of the animal precluded taking blood samples with each urine collection. The clearance value reported for each period is the average obtained from these multiple urine collections. A period of 1— hrs elapsed between each of two or three such clearance periods in 8 rats. The results of single clearance periods for another 5 rats are also reported. The renal artery was compressed with an adjustable screw clamp (Munnell and Gregg 1950) after the first clearance period in an additional 8 rats. These rats were allowed to recover for 1/2—1 hr after this procedure. At time when all the tubules were open and the urine flow rate was stable and approximately half the rat before clamping second clearance period was begun. Urine flow during arterial compression was low requiring 20—30 min urine collections, and the clearance is reported separately for each urine collection. Cin from the left kidney during arterial compression was estimated in 4 expts. from the infusion rate, plasma inulin concentration and Cin from the right kidney as well as being measured directly from the left ureteral urine flow in two of these experiments with flow greater than 1 µl/min.

Clearances from the left kidney are expressed in terms of kidney wight (k.w.) or doubled and expressed in terms of body weight (b.w.) The ratio of kidney weight to body weight decreases with increasing size of the rat (MacKay and MacKay 1927), but the number of nephrons remains constant until senescent degeneration begins (Smith 1951) Thus factoring Cin by body weight tends to reduce the calculated Cin per nephron in the heavier rats and narrow the range of Cin. The right kidney weight was used as the basis for expressing Cin from the left kidney in the eight rats whose left kidneys were frozen in situ. This approximation is justified by the observations that the right kidney weight differs from the left by only 0—10 % (Baines 1965, Arrizurieta, unpublished observations)

Photographs were taken through an 11× objective in a Leitz Ortholux microscope with an Ultropak® incident light illuminator without dipping cone. The selection of tubules for measurement was randomized by selecting three or four areas of the kidney surface, which were photographed at least three times each in the first clearance period. The same or immediately adjacent areas were re-photographed at last three times each during succeeding clearance periods. The photographic process further randomized the selection of tubules for measurement since it was impossible to predict which tubules would be in focus.

Photographs were enlarged to final magnification of 230—460× and the luminal diameter [illegible] d with calipers. Measurements were made in all the areas of proximal convolutions where the sides of the lumen were parallel and the white line of the brush border-fluid interface was clearly focused. Representative measurements are shown in Fig 1 Ten to thirty such measurements were made on each photograph and averaged. When a lumen narrowed or had narrow and broad segments, measurements of each segment were made and the average of these measurements was used to represent the diameter Fifteen to thirty photographs of each kidney were suitable for measuring, and between 150 and 350 measurements were made for each kidney The mean diameter for clearance period was obtained by averaging the means from all the photographs measured in that period. Measurements of the same tubule in 6—8 different pictures taken during the same clearance period had S.E. of between 0.6 and 0.8 µ. An analysis of variance of the results were done (Dixon and Massey 1957)

Diameters were measured by three observers, each selecting the sites to be measured. The results were usually in close agreement quantitatively and always showed the same qualitative variation from one period to the next and from animal to animal. In addition, 28 photographs were projected onto paper the tubular lumens outlined and subsequently cut out and weighed. The results also agreed quantitatively with the caliper measurements.

Results

In animals without arterial compression Cin varied either not at all or increased or decreased by as much as 0.4 ml/min g kidney weight (k.w.) between two clearance periods an hour or more apart. The mean Cin from 21 unclamped kidneys was 0.9 ml/min g k.w. (range 0.4—1.6) or 0.7 ml/min 100 g b.w. (range 0.4—1.4) for two kidneys (Fig 2) The mean Cin in 8 rats prior to renal artery compression was 1.2 ml/min g k.w. (range 0.8—1.6) or 1.0 ml/min 100 g b.w. (range 0.6—1.6)

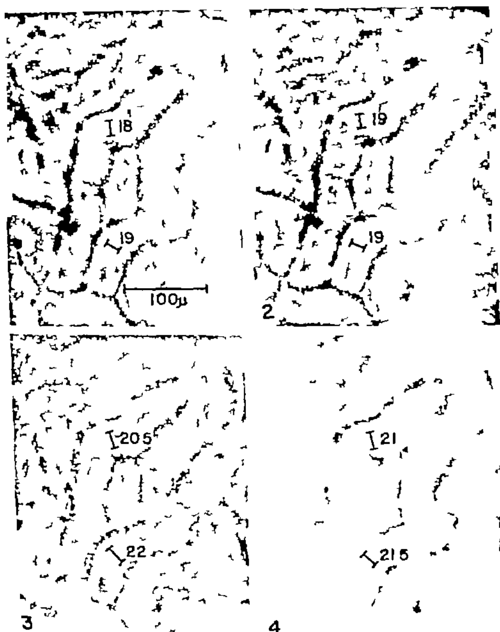


Fig. 1. Serial photographs of the surface of rat kidney with intact capsule. Two representative measurements at the same sites in each photograph are indicated by the black lines. Photographs 1 and were taken 30 min apart during the first clearance period (Cin 1.2 ml/min g k.w.). Mean diameter in 6 photographs of the same area as in this picture was 18.5 μ. Photograph 3 was taken 100 min after renal artery constriction (Cin 0.2 ml/min g k.w.). Mean diameter in 3 photographs was 21 . Photograph 4 was taken 130 min after clamping (Cin 0.3 ml/min g k.w. Mean diameter in 5 photographs was 19.5 . ×270)

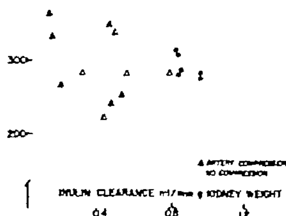


Fig. 2. Relationship between inulin clearance and mean cross sectional area of superficial proximal tubules. Single clearance periods from five rats and two clearance periods an hour or more apart from each of eight rats are shown by black dots. Results during partial renal artery compression shown by open triangles.

mean Cin after renal artery compression was 0.4 ml/min g k.w. (range 0.1—0.8) or 0.4 ml/min 100 g b.w. (range 0.1—0.9).

Tubular luminal diameters varied considerably within each kidney. No pattern to this scatter was detected. During an experiment some lumens would shrink and others expand. The range of luminal size was as great within one kidney as between different kidneys.

The mean proximal luminal diameter in all unclamped kidneys was 18.7 ± 1.5 μ (S.D.). In a single animal, the mean diameter remained constant or increased or decreased by as much as 2 μ during the course of an experiment. The mean difference between two periods without renal artery compression in a single rat was 1.2 μ. As shown in Fig. 2 luminal diameter was relatively constant and independent of Cin in the unclamped kidneys. An analysis of variance and t test were performed on the data from 8 rats in which two clearance periods an hour or more apart without arterial compression were measured. No correlation between Cin and diameter was found ($p > 0.8$).

Immediately following partial compression of the renal artery the proximal tubules collapsed and the kidney blanched, but blood flow did not stop. The tubules began to reopen 1—10 min later. Some convolutions regained their preclamped appearance rapidly while other remained narrow or occluded for 10—20 min. One-half to one hour later the kidney surface was indistinguishable from the preconstricted state, the blood supply appeared adequate, all but very occasional tubules had normal sized lumens, and the urine flow was stable. However, the tubules in

The absolute values of luminal diameters presented here should be taken with some reservation. They were measured between the inner borders of the what zone of the brush border fluid interface which may give an underestimate of the true diameter as will be discussed in further details in a subsequent paper.

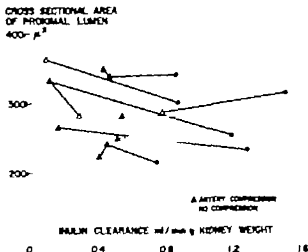


Fig. 3 Relationship between inulin clearance and mean cross sectional area of superficial proximal tubules before and after partial renal artery compression in eight rats.

these kidneys often collapsed momentarily after a dose of anesthetic was given or blood collected. This collapse rarely lasted more than 30 sec and care was taken to prevent its occurrence during a clearance period.

Cin decreased following renal artery constriction, but luminal diameters tended to increase (mean 19.5 ± 1 6 μ) (Fig 3) The increase was not significant according to a t-test of paired samples before and after arterial compression. In one rat the same area was clearly visible in 6 pictures from the control period and 8 from the two periods with constriction. The mean diameter for one specific convolution in these pictures was 17 7 ± 2.4 μ (S.D) in the control period and 21.8 ± 2.7 μ (S.D.) in the two periods when the artery was compressed. Th overall mean diameter from all photographs of this kidney increased from 18 μ in the control period to 21 and 19 in the two periods with arterial compression Cin decreased from 1 to 0 2 and then rose to 0.3 ml/min g k.w Four pictures of this area are hown in Fig 1

Discussion

We assume that volume changes in the superficial proximal con ol tions were representative of changes in all proximal tubules, th t there was no significant change in tubular length under the conditions of these experiments nd therefore that proximal luminal volume was linear function of the luminal radius squared.

The mean proximal luminal diameter of 18 7 in 21 nondiuretic rats without renal artery constriction was similar to that reported by other nvestigators (Leyssac 1963, Walter and Schoeppe 1963) Steinhausen *et al* (1963) measurements of luminal diameters (mean 22.8 μ) in decapsul ted kidneys of 200—400 g rats were slightly larger than ours. We observed in 4 rats that decapsulation resulted in an apparent increase in luminal diameters

Inulin clearances, which were similar to those previously found in nondiuretic rats (Leyssac 1963 Ullrich *et al.* 1963 Peters 1963) varied by as much as 0 4 ml/min g k.w in a single rat and 1.2 ml/min for the whole group. The observed urine flows of 0 001 to 0 005 ml/min are in the range to be expected from rats whose daily out put averaged 6 ml (Baines 1965 Walther and Schoeppe 1965) The absolute variations in GFR exceeded those in urine flow rate by a factor of as much as 10 to 200 indicating a direct proportionality between GFR and tubular reabsorption rate in these rats considered individually or as a group Although fractional reabsorption was not measured in these rats, F/P inulin ratios and occlusion times were measured in another small group of rats studied at the same time and under the same conditions (Leyssac and Gottschalk 1968) In this other group F/P inulin ratios (40—70 % of proximal tubular length) were 2.9 ± 0 9 (Mean ± S D) at Cin s between 0.65 and 0.95 ml/min g k.w and 3 0 ± 0.5 at Cin s of 1 0—1.2. These F/P inulin ratios are similar to those reported in other studies in which presumably Cin also fluctuated (Gottschalk 1963 Glabman *et al.* 1965) Proximal reabsorption rate estimated by the OT technique in the same small group varied directly with Cin (in the range 0 7—1 2 ml/min g k.w) as has previously been reported (Leyssac 1963, 1965) We assume therefore that proximal reabsorption rate varied directly with Cin in the group without renal artery clamping presented in this paper

According to some investigations the velocity of flow in superficial proximal convolutions measured by lissamine green transit time decreases when Cin is reduced by restricting renal arterial blood flow (Gertz *et al.* 1965 Alexander and Levinsky 1967) It seems logical to assume that GFR in the superficial nephrons decreases at least as much as overall Cin, especially in view of the fact that during hemorrhage cortical blood flow decreases relative to overall renal blood flow (Barger and Herd 1966) In unpublished experiments we confirmed the observation of reduced luminal velocity flow using lissamine green in vivo and ferrocyanide located as Prussian blue in microdissected nephrons. As expected ferrocyanide traveled less rapidly in superficial nephrons than in deep nephrons during renal artery compression. Lissamine green microinjected into proximal convolutions occasionally did not reappear in distal tubules following arterial compression. L g had appeared in these distal tubules after injection into the proximal tubule in the control state Flow had apparently stopped distal to the superficial proximal convolutions in these nephrons due to virtually total reabsorption of the filtrate Accordingly reduction of Cin below values of 0 6 ml/min g k.w may be explained in part by an increasing number of such noncontributory nephrons (Leyssac 1964 a) Judging from lissamine green and ferrocyanide transit times GFR in the superficial nephrons was probably low or lower than in the rest of the nephrons, and reabsorption may have been virtually complete by the end of the proximal tubule.

Even though the relationship between proximal reabsorption rate and Cin does not remain constant when the latter falls below 0.55 ml/min g k.w (0 45 ml/min 100 g b.w) the absolute rate of reabsorption per unit of tubular length either reaches a fixed minimum (Leyssac 1964 a) or continues to fall. An increase in late

proximal F/P inulin ratios to 5 1 in kidneys with clearances less than 0.55 ml/min g k.w indicated an increase in fractional reabsorption, although it was not large enough to maintain absolut overall reabsorption in the range observed at higher inulin clearances (Leyssac and Gottschalk 1968) However calculation of overall reabsorption rate from Cin and F/Pin cannot be exact when there is an unknown proportion of noncontributary nephrons. Previous measurements of the occlusion time at Cin below 0.55 ml/min g k.w suggested that reabsorption rate per unit of tubular length was at a fixed minimum in segments still ha ing an open lumen (Leyssac 1964 a, Leyssac and Gottschalk 1968)

In the present studies Cin was decreased 46—89 % by arterial compression from initial al es of 0.75—1 65 ml/min g k.w Absolute reabsorption rate must have been significantly reduced under these circumstances, although as the above discussion indicates the relation between reabsorption rate and Cin was not constant at very low clearance rates. Proximal luminal volume remained constant (Fig 3) Proximal luminal volume was also essentially unchanged in eight experiments in which Cin varied for unknown reasons by as much as 0 4 ml/min g k.w in an individual rat over a period of hours. Cin varied widely within the group of rats without arterial compression but luminal olume was relati ely constant (Fig 2)

Since the time of Smith it has been recognized that the "glomerulo-tubular balance shown by clearance experiments is a collective function of the entire nephron population. Th occlusion time technique gives an overall estimate of re absorption rate for a large area of the kidney surface. This technique has shown that glomerulotubular balance in nondiuretic rats is largely a property of the glomerulus and proximal tubule considered as a unit. In the present experiments photographs of numerous convolutions on the functioning kidney surface show that this balance was achieved without a significant correlation between GFR and average proximal tubular diameter either in the individual rat or in the group of rats. In addition these results confirm one of the assumptions upon which the interpretation of occlusion times rests—namely that the volume to be reabsorbed after clamping the renal artery is constant in nondiuretic rats undergoing spontaneous ariations in Cin and during partial renal artery compression. Thus the occlusion time is inversely proportional to the rate of net efflux of salt and water from the proximal tubule nondiuretic rats.

Under similar experimental conditions, probably associated w th a similar range of Cin, mean intratubular pressure did not differ significantly from one animal to another in a large group of rats (Gottschalk and Mylle 1956) Therefore if l minal pressure is the major determinant of the degree of tubular distension, one would not expect to find correlation between Cin and luminal olume Al h ange of pressures within one kidney was as great as the range w thin the total group of kidneys. Consistent with this observation w found the rang of l minal diameters within one kidney to be almost as great as that within the entire group

In a previous study on nondiuretic rats, compression of the renal rtery produced no alteration of proximal ntratubular pressure but halved distal ntratubular pres-

sure (Leyssac 1964 b) Thus it appeared that when Cin was reduced the pressure gradient from proximal to distal tubule increased while flow decreased therefore resistance to flow in the loop of Henle must have risen. An increased resistance to flow might occur when the lumen of the loop narrows and a point may be reached where interfacial tensions become important. A similar phenomenon in small blood vessels accounts for the "residual critical closing pressure" (Burton 1962) Pressures of 10—15 mmHg are required to overcome this resistance. The following sequence of events may explain our results in nondiuretic rats during arterial compression. Spasm of the renal artery induced by the clamping procedure produced an initial large drop in effective filtration pressure. The proximal tubules were seen to narrow or collapse and the thin limbs presumably also become narrower As arterial spasm lessened and probably intrarenal arterial resistance, effective filtration pressure and proximal intratubular pressure rose. Transiently most and sometimes all of the filtrate was reabsorbed before reaching the distal tubules and resistance to flow through the loop was high. When a steady state was attained, proximal intratubular pressure was maintained by the increased resistance to flow in Henle s loop implying that the loop by its structure was prevented from complete collapse due to the interfacial tension. In this state the reduction in glomerular capillary pressure was manifested by a decrease in distal intratubular pressure and Cin. With this alteration in the distribution of pressure the distal lumens were narrow while the proximal lumens were restored to their previous size.

The stabilizing influence of variable loop resistance on proximal luminal volume v have been lost in the hypertrophied diuretic kidneys studied by Rector *et al.* (1966 They observed proportional decreases in luminal volume and inulin clearance in hypertrophied diuretic kidneys following aortic clamping Even when Cin was reduced by aortic compression, the luminal diameters of diuretic hypertrophied nephrons in Rector s experiments were larger than those in nondiuretic nonh pertrophied nephrons Perhaps in dilated nephrons perfusion pressure and Cin can be decreased by relatively large amounts before the critical luminal size is reached at which resistance to flow increases significantly in the loops of Henle A reduction in glomerular capillary pressure in such nephrons would be manifested therefore by a decrease in intraluminal pressure and diameter in both proximal and distal convolutions of the nephron, since there would not be a high resistance segment separating them.

Steinhausen, Loreth and Olsen (1965) observed parallel decreases in tubular diameter and Cin 15—20 min after Cin was lowered by controlled bleeding They appear to have made their measurements before urine flow and glomerular and tubular function were stabilized. The reduction in tubular diameter may be similar to the transient partial or complete collapse of proximal tubules which we observed immediately following renal artery constriction. Our conclusions, howeve are based only on observations made 30—130 min after arterial compression, when all the visible tubules were open. In addition the loop of Henle may have been more dilated in their rats than in ours because of the diuretic effect of larger intravenous infu-

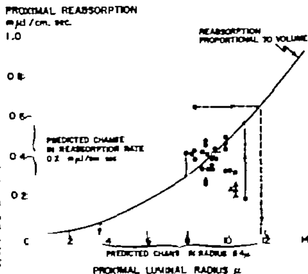


Fig. 4. Comparison of observed data with predicted variations if luminal radius and reabsorption rate for single tubule 1 cm long were related by the expression $0.41\ r^2/(9.7)$. The smooth curve shows the results predicted from this expression. The open circle indicates the mean radius and reabsorption rate for rats without arterial compression and Cin greater than 0.7 ml/min g k.w. Closed circles represent data from rats without arterial compression, and open triangles represent data from rats with arterial compression. See text for method of calculating reabsorption rate and extrapolating the expected results.

sion. No correlation between Cin and proximal diameter at arterial pressures between 100 and 140 mmHg is apparent in the data of Steinhausen *et al.*

We estimated the magnitude of the changes in reabsorption rate and luminal radius that should have occurred if they were related as Brunner *et al.* (1966) suggested. Reabsorption rate for a proximal tubule 1 cm long was calculated by assuming that 75 % of the filtrate was reabsorbed by the end of the proximal tubule and that there were 32 000 nephrons per gram kidney weight (Smith 1951). For reasons given above reabsorption was considered to be complete during arterial compression, although this gives an overestimate of the reabsorption rate. A curve relating luminal volume to reabsorption rate was calculated in a similar fashion to that shown by Brunner *et al.* (1966 Fig. 3). Using the mean radius of 9.7 μ and mean reabsorption rate of 0.41 mμl/sec.cm for rats without arterial compression and with Cin greater than 0.7 ml/min g k.w. the reabsorption rate per cm at any luminal radius (r) should be calculated as $0.41\ r^2/(9.7)$ (Fig. 4). Using this relationship the predicted range of radii can be extrapolated from the observed range of reabsorption rates. The predicted change in radius of 8.4 μ was not observed although the method was sufficiently sensitive to detect a much smaller change. The change in reabsorption rate predicted for a single tubule from the observed change in radius is 0.21 mμl/cm sec or 0.50 ml/min for the whole kidney. It is evident that reabsorption rate fluctuated by much more than this amount in individual rats and in the group as a whole. For the group without arterial compression and with clearances greater than 0.7 ml/min the predicted change in radius was 4.4 μ and the predicted change in reabsorption rate was 0.40 ml/min for the whole kidney. Again these changes could have been detected but were not observed.

It might still be argued that reabsorption rate in these nondiuretic rats was determined by a combination of volume and hormonal effects or as Rector *et al.* (1967)

have stated that inhibition of intrinsic cellular reabsorptive capacity alone did not reduce overall reabsorption rate. Some of the evidence for this argument is examined in the following paragraphs.

Proximal reabsorption rates calculated by Rector *et al.* (1966) from transit times and F/P inulin ratios were higher in rats with elevated ureteral pressures and dilated proximal tubules than in control rats. However when proximal reabsorption rates are calculated from the inulin clearances and F/P inulin ratios in their Table V there appears to be no difference between their two groups of rats, despite presumed large differences in luminal diameter. The discrepancy between these two indirect estimates of proximal reabsorption rate leaves its numerical value in doubt.

Both Gertz *et al.* (1965) and Rector *et al.* (1966) reported a linear relationship between F/P inulin ratios and transit times in single oil-blocked tubules when Cin was reduced by increasing ureteral pressure and/or altering arterial pressure. Both groups of investigators have used this correlation to support the theory that proximal reabsorption rate is causally related to luminal volume in such a way that $C/\pi r^2$ is constant (cf. equation 1).

Equation 1 $\ln (TF/P)_{In} = T\ C/\pi r^2$

Where C = reabsorption rate in mμl/sec/cm T = transit time in sec r = radius in μ and πr^2 = tubular volume per unit of tubular length (Brunner *et al.* 1966). An alternative possibility is that both C and r^2 were unchanged in the tubules examined and that F/P inulin varied with transit time. Since luminal diameters and filtration rates were not reported for the tubules from which fluid was collected for F/P inulin determinations (Rector *et al.* 1966), it is impossible to calculate their absolute rate of reabsorption. Moreover the diameter and filtration rate of an oil-blocked tubule may well depend more upon the sampling technique than upon the functional state of the rest of the kidney. As mentioned above, according to one method of estimation, proximal reabsorption rate in the studies of Rector *et al.* was unchanged by elevating ureteral pressure. The scatter of results in measurements of F/P inulin and transit time (Rector *et al.* 1966 Fig. 3) is sufficiently wide that variations in reabsorption rate independent of luminal volume such as those described in our paper might not be detected.

Wiederholt *et al.* (1967) found a correlation between water reabsorption rate and tubular volume in isolated perfused proximal tubular segments. Pump-perfusion led to relative distension of the proximal tubule (Windhager, personal communication) which suggests that the perfused tubules were not analogous to intact tubules, which can accommodate marked increases in steady state flow without dilatation, as in the present experiments. In microperfusion experiments the transit time may be largely determined by the arbitrarily selected infusion and withdrawal rates. Prolongation of transit times would explain the unusually high F/P inulin ratios observed in their experiments (average F/P inulin ratio was 2.3 at a distance of 2000 μ from the injection site.

The arguments outlined above and the results of our experiments do not rule out

the possibility of a relationship between volume and reabsorption rate, particularly in isolated oil-blocked tubules. Such tubules are more dilated than undisturbed tubules in nondiuretic rats, and a relation between volume and reabsorption may only be demonstrable under these conditions of dilatation and obstruction.

In conclusion, the degree of tubular distension apparently did not determine proximal reabsorption rate in nondiuretic rats since proximal luminal diameter remained relatively constant when Cin fluctuated spontaneously or was decreased by partial artery compression.

References

Alexander, E. A. and N. G. Levinsky. Nephron function during hypotension. Persistence of glomerular filtration in anuria. Proc. 50th Ann. Meet. Amer. Soc. Clin. Invest. *J. clin. Invest.* 1967. *46.* 1032.

Baines, A. D. *Thesis.* University of Toronto, 1965.

Barger, A. C. and J. A. Herd, Study of renal circulation in the unanesthetized dogs with inert gases. 1) External counting. *Proc. III Int. Congr. Nephrology.* Washington. D.C. S. Karger. Basel 1967. *1.* 174—187.

Brunner, F. P., F. C. Rector, J. and D. W. Seldin, Mechanism of glomerulotubular balance. II. Regulation of proximal tubular reabsorption by tubular volume, as studied by stopped flow microperfusion. *J. clin. Invest.* 1966. *45.* 603—611.

Burton, A. C., Physical principles of circulatory phenomena. The physical equilibria of the heart and blood vessels. In: *Handbook of Physiology.* Circulation Sect. 2. Vol. 1. Ed. W. F. Hamilton, Philip Dow. Amer. Physiol. Soc. 1962. 85.

Dixon, W. J. and F. J. Massey, J. *Introduction to statistical analysis.* 2nd Ed. McGraw Hill 1957.

Gertz, K. H., J. A. Mangos, G. Braun and H. D. Pagel, On the glomerular tubular balance in the rat kidney. *Pflügers Arch. ges. Physiol.* 1965. *285.* 360—372.

Giebisch, G. and E. E. Windhager, Renal tubular transfer of sodium, chloride and potassium. *Amer. J. Med.* 1964. *36.* 643—669.

Glabman, S., H. A. Aynedjian and N. Bank, Micropuncture study of the effect of acute reductions in glomerular filtration rate on sodium and water reabsorption by the proximal tubules of the rat. *J. clin. Invest.* 1965. *44.* 1410—1416.

Gottschalk, C. W. Renal tubular function. Lessons from micropuncture. *Harvey Lect.* Series 58, 1963. 99.

Gottschalk, C. W. and P. P. Leyssac, Proximal tubular function in rats with low inulin clearance. *Acta physiol. scand.* 1968. *74.* 453—464.

Gottschalk, C. W. and M. Mylle, Micropuncture study of pressures in proximal tubules and peritubular capillaries of the rat kidney and their relation to ureteral and renal venous pressures. *Amer. J. Physiol.* 1956. *185.* 430—439.

Hanssen, O. E. A histochemical method for evaluation of excreted ferrocyanide in isolated tubules of the mouse kidney. *Acta path. microbiol. scand.* 1958. *44.* 363—371.

Lassiter, W. E., M. Mylle and C. W. Gottschalk, Net trans-tubular movement of water and urea in saline diuresis. *Amer. J. Physiol.* 1964. *206.* 669—673.

Leyssac, P. P. Dependence of glomerular filtration rate on proximal tubular reabsorption of salt. *Acta physiol. scand.* 1963. *58.* 236—242.

Leyssac, P. P. The in vivo effect of angiotensin on the proximal tubular reabsorption of salt in rat kidneys. *Acta physiol. scand.* 1964 a. *62.* 436—448.

Leyssac, P. P. The effect of partial clamping of the renal artery on pressures in the proximal and distal tubules and peritubular capillaries of the rat kidney. *Acta physiol. scand.* 1964 b. *62.* 449—456.

Leyssac, P. P. The in vivo effect of angiotensin and noradrenaline on the proximal tubular reabsorption of salt in mammalian kidneys. *Acta physiol. scand.* 1965. *64.* 167—175.

MacKay, L. L. and E. M. MacKay. Factors which determine renal weight. II. Age. *Amer. J. Physiol.* 1927. *83.* 191—195.

Mitchell, E. R. and D. E. Gregg, A small adjustable renal artery clamp for production of chronic hypertension in the rat. *Proc. Soc. exp. Biol. (N.Y.)* 1950. *73.* 645.

Peters, G., Compensatory adaption of renal function in the unanesthetized rat. *Amer. J. Physiol.* 1963. *205.* 1042—1048.

RECTOR, F. C., JR., F. P. BRUNNER and D. W. SELDIN: Mechanism of glomerulotubular balance. I. Effect of aortic constriction and elevated uretero-pelvic pressure on glomerular filtration rate, fractional reabsorption, transit time and tubular size in the proximal tubule of the rat. *J. clin. Invest.* 1966, *45*, 590—602.

RECTOR, F. C., JR., J. C. SELLMAN, M. MARTINEZ-MALDONADO and D. W. SELDIN: The mechanism of suppression of proximal tubular reabsorption by saline infusions. *J. clin. Invest.* 1967, *46*, 47—56.

SMITH, H. W.: *The Kidney*. Oxford Univ. Press, New York, 19 1, pp. 1049.

STEINHAUSEN, M., L. IRAVANI, G. E. SCHUBERT and R. TAUGNER: Auflichtmikroskopie und Histologie der Tubulusdimensionen bei verschiedenen Diuresezuständen. *Virchows Arch. path. Anat.* 1963, *336*, 503—527.

STEINHAUSEN, M., A. LORETTI and S. OLSON: Messungen des Tubulären Harnstromes, seine Beziehungen zum Blutdruck und zur Inulin-Clearance. *Pflügers Arch. ges. Physiol.* 1965, *286*, 118—141.

ULLRICH, K. J., B. SCHMIDT-NIELSEN, R. O'DELL, G. PEHLING, C. W. GOTTSCHALK, W. E. LASSITER and M. MYLLE: Micropuncture study of composition of proximal and distal tubular fluid in rat kidney. *Amer. J. Physiol.* 1963, *204*, 527—531.

WALTHER, D. and W. SCHOEPPE: Der Einfluss des intratubulären Druckes auf die Tubulusweite unter normalen und pathologischen Bedingungen. *Klin. Wschr.* 1965, *43*, 1092—1094.

WIEDERHOLT, M., K. HIERHOLZER, E. E. WINDHAGER and G. GIEBISCH: Microperfusion study of fluid reabsorption in proximal tubules of rat kidneys. *Amer. J. Physiol.* 1967, *213*, 809—818.

Acta physiol. scand. 1968. 74. 453—464

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Proximal Tubular Function in Rats with Low Inulin Clearance

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Abstract

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Proximal tubular salt and water reabsorption were studied in relation to the inulin clearance (Cin) in anesthetized rats maintained on a standard diet with and without partial clamping of the renal artery and in rats on a sodium deficient diet. The rate of proximal reabsorption, determined as the reciprocal of the occlusion time (OT), was linearly related to Cin above clearance values of 0.7 ml/min g kidney weight (k.w.). Below this value, proximal reabsorption rate reached a fixed minimum corresponding to an OT of 25—26 sec. Late proximal fluid to plasma inulin ratios averaged 3.0 at Cin greater than 0.65 ml/min g k.w. and 5.1 when Cin was 0.55 ml/min or less. This significant difference in fractional reabsorption was predicted from the OT measurements. Factors leading to a decrease in caliber of the proximal tubular lumen following retrograde microinjection of the distal convolution of the same nephron were also studied. When seen, proximal collapse appeared to be due to leakage of tubular fluid at a previous proximal puncture site and did not occur when procedures were taken to minimize leakage.

The rate of reabsorption of salt and water in the proximal tubule of nondiuretic rats has been shown in studies using the occlusion time technique to vary in direct proportion to the glomerular filtration rate over a range of inulin clearances (Cin) from 1.7 to 0.7 ml/min g kidney weight (k.w.). Below this range proximal reabsorption rate per unit of tubular length appeared to reach a fixed minimum. The luminal cross sectional area of superficial proximal convolutions remained unchanged or even increased slightly at low Cin, presumably due to an increased resistance to flow in some more distal segment(s) of the nephron, probably the thin limb of Henle's loop (Leyssac 1963, 1964 a and b, 1965, Baines et al. 1966).

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These observations lead to the predictions that 1) proximal fractional reabsorption should increase when Cin decreases below an absolute value of about 0.6 ml/min g k.w. and 2) that at low levels of filtration a further decrease in filtered load (in the sense of pressure in Bowman's capsule) increase in reabsorption or leakage of fluid from the proximal lumen would result in a decrease in luminal diameter or complete collapse of the proximal tubule. Either of these latter possibilities, i.e., increase in reabsorption or leakage are alternative explanations for the proximal collapse reported by Thurau and Schnermann (1965) to occur following distal microinjection of saline.

These predictions are confirmed by the results of the present study which demonstrate 1) that proximal fractional reabsorption of salt and water increases significantly when Cin decreases from above 0.65 to below 0.55 ml/min g k.w., and 2) that leakage of tubular fluid out of a proximal puncture site may result in proximal tubular collapse in kidneys with low inulin clearances. Furthermore, proximal tubular collapse following distal retrograde microinjection occurred only under conditions favoring a leakage artifact and was prevented by procedures taken to minimize leakage.

Under conditions of low Cin and luminal flow rate the resistance to movement of an injected oil droplet becomes significant and partial or complete tubular collapse is likely to result from fluid withdrawal, even though the position of the oil droplet remains unchanged. Too rapid fluid collection, thus may have influenced the results of previous micropuncture studies of proximal fractional reabsorption under such conditions. In the present investigations the luminal diameter rather than the position of an oil droplet was used as the reference to the rate of tubular fluid collection.

Methods

Male white rats of the Wistar strain weighing 200–300 g and allowed free access to food and water prior to the experiment were anesthetized by intraperitoneal injection of sodium amobarbital 5 mg/kg body weight b.w. or pentobarbital, 50 mg/kg, and prepared for micropuncture and inulin clearance (OT me surement as previously described Leyssac 1963, 1964 a, Gottschalk and Mylle 1956. The kidney was held in plastic cup only on rare instances when required to control excessive movements synchronous with respiration, and its capsule was not removed except when specifically stated. Rats fed standard diet were given 1.0–1.5 ml of 0.9 % saline intravenously during the time of preparation approximately 30 min. Salt-depleted rats were given 0 .0 ml of 9 saline during this period.

Inulin clearance and fractional water reabsorption were measured using tritiated inulin. A priming dose of 4 μc was given in 0.3 ml of normal saline and was followed by continuous i.v. infusion at 0.00 ml/min containing 1.4 μc/min of inulin-H. At least 45 min elapsed between the priming injection and the beginning of the Cin clearance period. An arterial blood sample was drawn before and after each clearance period which included two or three equal urine collections of 5–20 min duration. In a few experiments only one urine collection of at least 30 min duration was obtained. Twenty five units of heparin were injected before the first blood sample was collected. Radioactivity in plasma, urine and tubular fluid was measured as previously described (Leyssac et al. 1964). Cin was calculated both on the basis of k.w. and b.w. For the former the kidney was drained of fluids before weighing. For the latter the clearance of the experimental kidney was doubled.

For fluid/plasma (F/P) inulin determination tubular fluid was collected from convolutions demonstrated by microinjection of lissamine green to be the last surface convolutions of proximal tubules. Fluid samples were collected very slowly, up till more than 60 min for the collection of about 30 nano-l and great care was taken to adjust the withdrawal of fluid to the rate

at which there was no detectable change in luminal diameter. This proved a more satisfactory guide to rate of collection than a constant position of an injected oil droplet since especially in clamped, non-diuretic rats and in salt depleted rats the position of an injected oil droplet at times remained constant even though the rate of collection resulted in partial or complete collapse of convolutions proximal to the site of puncture. Also an injected oil droplet at times maintained a constant position in tubule even though the collection of fluid was temporarily interrupted and there was no apparent leakage at the site of puncture. The kidney was subsequently macerated and the locus of the puncture site was identified in the microdissected nephron and its distance from the glomerulus was measured with an ocular micrometer.

In microinjection experiments proximal and distal convolutions belonging to the same nephron were identified by proximal intratubular injection of a small amount of 2 % lissamine green in saline. The following solutions colored with lissamine green were used for retrograde distal microinjection: 1) sodium chloride 300 mM, 2) sodium chloride 150 mM, and 3) mannitol 300 mM. For retrograde injection into proximal tubules, sodium chloride 150 mM was used. Changes in the proximal convolutions of the injected and adjacent nephrons were observed visually or photographically. Photomicrographs of the injected nephron were taken through the side arm of an A. O. Spencer stereoscopic microscope at 80x magnification using a Robot Star II automatic camera synchronized with a Leitz electronic flash unit. Photographs were exposed automatically at 6 sec intervals on black and white film (Kodak Plus X).

Microinjection and collection experiments were performed on three groups of rats: 1) control nondiuretic rats fed a standard diet (Purina Laboratory Chow), 2) nondiuretic rats fed a standard diet and in which the renal artery was compressed by a small adjustable screw clamp (Munoel et al. 1950) and 3) nondiuretic rats fed a synthetic sodium-deficient diet and distilled water for 4—12 weeks prior to the experiment (Nutritional Biochemicals Corp. Sodium Deficient Test Diet).

Results

A. Occlusion time experiments

Occlusion times and/or inulin clearances were measured in 2? salt-depleted and 25 nondiuretic control rats. Clearances and occlusion times were also measured in 8 of the control rats while the renal artery was partially constricted. The results of experiments in which both Cin and OT were measured are presented in Fig. 1. As had been found previously (Leyssac 1963, 1965) there was a linear relationship between

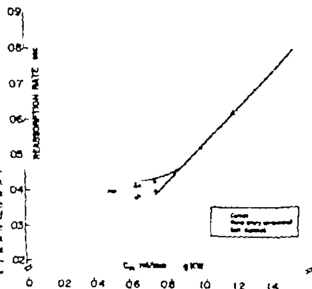


Fig. 1. Relationship between inulin clearance and reabsorption rate of salt determined as the reciprocal of the occlusion time in seconds. The diagonal and horizontal regression lines are previously determined from data obtained under control conditions (y = 0.333x) and after administration of angiotensin (y = 0.139 0.004) respectively.

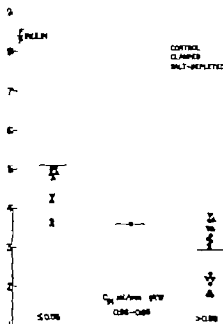


Fig 2. Last proximal inulin F/P ratios determined at various inulin clearances.

Cin and proximal reabsorption rate of salt and water determined as 1/OT above clearance values of 0 7 ml/min g k.w Below a Cin of approximately 0.7 ml/min g k.w the reciprocal of the occlusion time (the proximal reabsorption rate per unit of tubular length) reached a fixed minimal value corresponding to an occlusion time of 25—26 sec. Cin under control conditions averaged 0.99 ml/min g k.w (0.74 ml/min 100 g b w) Most of the inulin clearances from salt-depleted rats fell between 0.5—0 8 ml/min g k.w and averaged 0 73 ml/min g k.w (0.57 ml/min 100 g b.w)

B. *Tubular fluid collection experiments*

Tubular fluid was collected for inulin determination from the last accessible convolution of 18 proximal tubules of 16 salt-depleted animals and 22 tubules of 11 normally fed nondiuretic rats with or without renal artery compression. F/P inulin ratios *in these experiments are presented in Fig 2 grouped according to the inulin clearance.* The mean F/P inulin ratio was 3 0 for 20 samples obtained at an inulin clearance above 0 65 ml/min g k.w (site of puncture averaged 55 % of proximal length) The mean ratio was 3 6 for 6 samples obtained at a Cin between 0.56 and 0 65 ml/min g k.w (site of puncture averaged 50 % of proximal length) and was 3 1 for 14 samples collected at clearance levels less than 0.55 ml/min g k.w (puncture site averaged 54 % of proximal length) Fluid was obtained at the same puncture site in 5 of these tubules before and after Cin was reduced on the average from 0 88 to 0.31 ml/min g k.w by renal artery compression. The F/P inulin ratios without arterial compression were 3 3 2.2, 3 1 4 6 and 3 4 after compression they were 5.0, 4 9 4.9 5.8, and 3 4 respectively

Analysis of variance between these three groups showed that the increase in the F/P inulin ratio observed when Cin decreased from above 0.65 to below 0.55 ml/min g k.w is highly significant at the 0.1 % level (variance ratio F = 14.3 37 degrees of freedom) Even when the two extremely high F/P inulin ratios in the group with lowest Cin are excluded the increase is still highly significant at the 0.1 % level (F = 11 7) Using Scheffé's method of multiple comparison (1952) the two extreme groups differed significantly at the 0 1 % level, but the intermediate group (Cin between 0.56 and 0 65 ml) did not differ from either extreme even at the 1 % level.

A correlation between F/P inulin ratios and inulin clearances was not detected at clearance levels above 0.65 ml/min g k.w The F/P ratios averaged 2.9 ± 0.9 (SD) at Cin between 0.65 and 0.95 ml/min g k.w and 3.0 ± 0.5 (SD) at Cin of 1 0—1.2 ml/min. However these experiments were designed to obtain data at low clearance values and the ratios obtained at Cin above 0.65 ml/min are few—20— and do not encompass the entire range of variation of Cin observed in nondiuretic rats.

C. *Distal microinjection experiments*

The results of the distal retrograde microinjection experiments are presented in Table I In kidneys with intact capsules a change in proximal diameter was seen in only 7 out of a total of 76 expts. Complete collapse of reacting convolutions was observed in only three of these seven experiments. In 43 of these experiments 300 mM NaCl was injected, and in the remaining 33 expts., 150 mM NaCl. Even though the proximal diameter usually increased during retrograde distal injection, no reflux of the colored injectate into the proximal convolution was ever observed.

When the kidneys were decapsulated, proximal collapse occurred more frequently and when a larger pipette (5—7 μ at the tip) was used for the preceding proximal puncture it occurred consistently in decapsulated kidneys. Those proximal convolutions which collapsed following distal microinjection of sodium chloride usually also collapsed following distal microinjection of mannitol solution. Collapse was not seen in the three nephrons in which proximal reflux during distal injection of mannitol was observed. In contrast to gradual occlusion over a period of 20—26 sec observed in salt-depleted kidneys when their circulation was abruptly interrupted by aortic clamping for measurement of occlusion time proximal collapse after distal microinjection was sudden and followed transient distension. Collapse was complete within 10—15 sec after the start of the distal injection, and the tubule generally reopened within 30—60 sec. After reopening, the luminal diameter often appeared somewhat smaller than prior to injection. In six experiments in which a small amount of lissamine green was injected proximally and distal retrograde injection begun as soon as the dye had disappeared into the pars recta, dye reflux into the proximal convolutions was always seen with proximal collapse (Fig 3) In one experiment reflux extended proximal to the puncture site and visible leakage of dye occurred through the proximal puncture site at the time of collapse

To exclude the possibility that proximal collapse was mediated by the juxtaglomerular apparatus (JGA) proximal tubules of decapsulated kidneys of salt-depleted

TABLE I Summary of results of distal microinjection experiments

Sol tio Injected distally[1]	Number of observa-tions	N mber of proxi-mal tubu-les col-lapsing	Tip size for proximal puncture	N mber showing proximal reflux	Comments
Capsule intact.	Renal artery compressed.				
N Cl	33	4	3—5 μ	0	Collapse complete in 1 t bule only
Capsule intact. Salt-depleted.					
N Cl	43	3	3—5 μ	0	Collapse complet 1 2 tubules only
Decapsulated. Salt-depleted. Reinjections w th different solutions.					
N Cl	13	8	3—4 μ	0	1st injection
NaCl	5	2	3—4 μ	2	2nd injection into the non-reactive nephrons t higher pressure
M nnitol	1	0	3—4 μ	0	3rd injection into 1 of the non-reactiv nephrons
M nnitol	7	0	3—4 μ	0	1st injection
N Cl	7	0	3—4 μ	0	2nd injection
Cl	7	7	5—7 μ	0	1st injection
nnitol	7	6	5—7 μ	0	2nd injection
N Cl	3	3	5—7 μ	0	1st injection
Mannitol	3	0	5—7 μ	3	2nd injection

NaCl refers t both 300 nd 150 mM sol tions
Mannitol refers to 300 mM solution

rats were filled with a long column of mineral oil downstream to the puncture site. In 4 expts. pipettes 3—4 μ in tip diameter were used for injection of mineral oil and subsequent proximal retrograde injection of saline Following removal of the pipette the tubules collapsed and afterwards reopened in all 4 expts. Similar experiments were performed on 6 proximal tubules using pipettes with tip diameters of 6—7 μ. All six tubules collapsed after retrograde injection and removal of the pipette, but none reopened (Fig 4) Moreover n an additional 5 expts. with 6—7 μ pipettes, proximal tubular collapse occurred after puncture and removal of the pipette even without injection of mineral oil or saline The obvious explanation of these results is that fluid leaks out of tubule following removal of a micropipette and the larger the puncture hole, the greater the leakage and the lesser the likelihood that the puncture site will seal off

Fig. 3 Serial photomicrographs of kidney surface showing proximal reflux of distally injected dye. Photograph A was taken during microinjection of lissamine green into proximal convolution (P); dye has appeared in the distal convolution (D). In B retrograde injection into the distal convolution has begun. C and D demonstrate dye reflux into proximal convolutions.

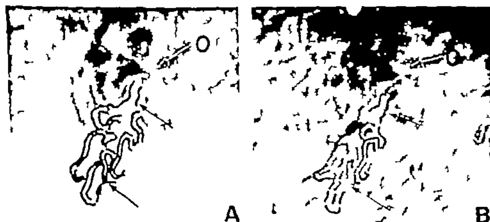


Fig. 4 Photomicrographs showing collapse of proximal tubule blocked downstream to the puncture site by an oil column (O). *A. retrograde proximal injection of lissamine green through pipette.* B. Pipette removed and proximal convolutions collapsed. Arrows point to the same convolutions before and after collapse.

Discussion

A. *Fractional water reabsorption*

The large scatter of F/P inulin ratios under any given physiological condition reported by all investigators probably results from functional differences between individual nephrons as well as technical errors. Particularly troublesome is adjustment of the rate of collection so that the tubular flow rate is neither accelerated nor impeded when a relatively large volume of tubular fluid is collected for analysis, as is the case for inulin determinations. Generally the rate of withdrawal of fluid is adjusted so that an injected oil droplet maintains a constant position in the tubular lumen. Inherent in this experimental technique are two conflicting requirements: 1) the mobility of the droplet must be unrestricted, yet 2) the contact between the tubular wall and oil must be so tight that leakage of fluid in either direction past the oil blockade is impossible. Difficulties with these requirements become critical under such diverse conditions as distal collections during osmotic diuresis and proximal collections at low filtration pressures. Partial or complete collapse of the tubule distal to the oil drop may reasonably be expected during proximal collection when the volume flow and pressure in the distal convolution are low, as during renal artery compression and salt depletion, and will add to the forces preventing movement of the oil droplet downstream. In preliminary experiments in kidneys under these conditions we observed that an oil droplet at times would not move downstream although no fluid was being collected. At times the surface of the oil drop could be observed to undulate, suggesting leakage past it. Nor would the oil droplet necessarily move upstream towards the pipette when fluid was collected too rapidly. Instead one or more convolutions of the tubule collapsed partially or completely. This should increase the effective filtration pressure of the punctured nephron, and its filtration rate would no longer be representative of that of the entire kidney (Cin) and the F/P inulin ratio may be falsely low. Thus, under these conditions the position of an injected oil droplet is a poor reference for rate of collection. For these reasons an oil blockade was not used in the present experiments, but fluid was collected at a very slow rate in an effort to prevent changes in intratubular pressure. Fluid was not collected retrograde since only some unknown percent of fluid (about 10—15 %) flowing past the pipette was collected and flow downstream continued. Despite these precautions, at low inulin clearances some decrease in luminal diameter was occasionally produced indicating a reduction in proximal intratubular pressure. Since this may have accelerated proximal flow rates, we suspect that our F/P inulin ratios are spuriously low at low Cin.

The F/P inulin ratios were significantly greater at low than at high inulin clearances and averaged 3.0 at Cin above 0.65 ml/min/g k.w. and 5.1 when Cin was less than 0.5 ml/min. Between these levels of Cin, the F/P inulin ratio averaged 3.6, a value not significantly different from that found at either higher or lower Cin.

These results were as expected from experiments utilizing the occlusion time technique, which indicated that the proximal rate of reabsorption varies in direct pro-

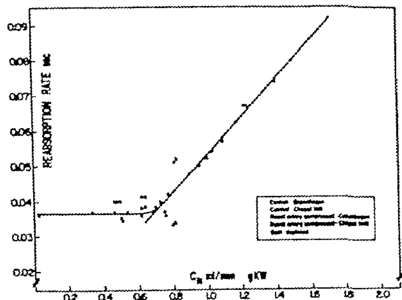


Fig. 5. Relation between inulin clearance and proximal reabsorption rate of salt (I/OT). Mass plot of data obtained in the present experiments and previously reported data (Leyssac 1963, 1965)

portion to the filtration rate over a range of Cin from approximately 0 6 to 1.8 ml/min g k.w but remains unchanged at a fixed low value when Cin is less than approximately 0.6 ml/min. Fig 5 indicates that results obtained on rats with this technique in Chapel Hill agree well with those obtained on a larger number of rats in Copenhagen.

In the present studies on nondiuretic rats the proportionality between proximal reabsorption rate (I/OT) and Cin was maintained over a range of variation amounting to a factor of 2 to 2.5 Since the absolute rate of proximal reabsorption may increase by a factor of 2 to 2 5 with constant fractional reabsorption during hypertonic loading in the rat (Lassiter *et al.* 1961 Giebisch *et al.* 1964) it is reasonable to predict that inulin clearance may be halved by renal arterial clamping during hypertonic loading without significantly affecting the proximal F/P inulin ratio

The present results are in general agreement with unpublished studies in this laboratory on rats expanded with isotonic saline which exhibited increased proximal F/P inulin ratios after renal artery compression even with relatively small reductions in inulin clearance. The absolute rate of proximal sodium reabsorption is diminished by isotonic loading and—assuming that there exists a minimal proximal tubular reabsorption rate—increased fractional reabsorption is to be expected following relatively small decreases in filtration rate

Our observations differ from those of Glabman, Aynedjian and Bank (1965) who reported no increase in fractional reabsorption with reductions of Cin up to

66 % even though the control clearance values were in the low range of normal (re calculated to approximately 0.5 ml/min 100 g b.w.). Fractional reabsorption rose in two rats in which the inulin clearance was reduced by more than 90 %. Although indicating a general relationship between filtration and reabsorptive rates, we do not believe that their results exclude significant increases in fractional reabsorption with reduced inulin clearance. They were not recollection experiments, and the large scatter in their results and the possibilities of technical difficulties in collection of tubular fluid during aortic compression mentioned above may account for the discrepancies between their results and ours.

Gertz *et al.* (1965) found directly measured and estimated proximal fractional reabsorption in normally hydrated nondiuretic rats to increase when arterial pressure was reduced. They concluded that this increase resulted from increased transit time since reabsorptive capacity determined by the split oil droplet method was constant and independent of renal blood flow and Cin. These authors did not relate individual F/P inulin ratios to corresponding Cin values, complicating a direct comparison of their results with ours. It appears, however, that there was no significant increase in F/P inulin ratios until Cin was reduced below 0.5 ml/min 100 g b.w., largely in accordance with our observations.

Brunner, Rector and Seldin (1966) reported that aortic compression had a different effect in unilaterally nephrectomized rats depending upon their pretreatment. In rats given 5—6 ml of isotonic saline during their operative preparation, reduction in arterial pressure to 60—65 mm Hg resulted in an average decrease of 28 % in tubular volume and of 48 % in Cin from a control average of 9.5 ml/min kg b.w., but proximal transit time, halftime for droplet reabsorption and estimated fractional reabsorption were all unchanged. In rats not given saline during their operative preparation, control Cin averaged 5.4 ml/min kg b.w. and a decrease in arterial pressure to 80—85 mm Hg was associated with a 65 % reduction in Cin and increased transit time, halftime for droplet reabsorption and estimated fractional reabsorption. Luminal volume was not significantly reduced. Furthermore, Rector, Brunner and Seldin (1966) found no change in proximal F/P inulin ratios in recollection experiments in 8 tubules of two rats undergoing isotonic saline diuresis when the GFR was reduced by 37 or 5 % from a value of 11.5 and 12.2 ml/min kg b.w., respectively. They concluded from their observations that proximal tubular reabsorption is a function of tubular volume and that glomerulotubular balance is maintained when tubular volume falls in proportion to reduction in GFR. But as Brunner and associates (1966) point out, the proportionality between rate of tubular reabsorption and luminal volume did not always hold for reasons not apparent.

The observations of the Dallas group (1966) in respect to halftime of droplet reabsorption thus did not agree with those of Gertz and co-workers (1965), whereas their transit time measurements agreed when the experimental conditions were similar. The estimates of fractional reabsorption of both groups are in general agreement with our predictions from OT experiments but the mechanism they postulate in explanation of this relationship is totally different.

In the dog available data indicate that fractional reabsorption is unchanged following reduction of inulin clearance by arterial compression. Using the recollection technique Dirks, Cirksena and Berliner (1965) reported that the decreased fractional reabsorption seen in dogs following isotonic loading was not prevented by aortic clamping during saline diuresis. The possibility of too rapid fluid collection during clamping is suggested by their statement that "after clamping the samples were collected almost as rapidly as in the diuretic phase without clamping" even though the tubules were noted to be narrower and paler and the kidneys smaller and softer. It is also conceivable that continued loading with saline may have obscured an effect of reduction in filtration rate on the measured F/P ratios. This possibility is emphasized by their observation that F/P inulin ratios occasionally failed to decrease in the first recollection period during saline infusion but were uniformly decreased in later collection periods during arterial compression. Watson (1966) came to conclusions similar to those of Dirks and associates (1965) from studies during isotonic saline diuresis in dogs, but his data appear less significant because of the large scatter in results and because values in clamped and unclamped periods were obtained not only in different tubules but in different animals.

B *Microinjection experiments*

Thurau and Schnermann (1965) reported microinjection experiments in salt depleted rats that appeared to be the first direct evidence in favor of a feedback mechanism controlling distal sodium load operating through the juxtaglomerular apparatus. They found that retrograde microinjection of saline (isotonic or hypertonic) into distal convolutions of decapsulated kidney was followed by complete collapse of the proximal convolutions of the same nephron 10 sec after start of the injection. Proximal and distal convolutions of the same nephron had previously been identified by proximal injection of lissamine green. Proximal collapse was not seen after distal microinjection of either mannitol solution in saltdepleted rats or saline injection in rats given 1% sodium chloride solution for drinking. After salt-depletion Cin is low and proximal fractional reabsorption tends to increase and a small but sudden loss of tubular fluid for any reason will result in proximal collapse. Thus, theoretically the collapse observed by Thurau and Schnermann could have resulted not only from reduction in filtration rate (or rather glomerular capillary pressure) as they postulated, but also from leakage at the proximal puncture site or from increased rate of reabsorption. As the collapse was sudden and complete within 10 sec and since the proximal occlusion times is twice as long after aortic clamping in salt depleted rats (Fig. 1) leakage of fluid, or less likely increased rate of reabsorption would appear to have been the probable cause of the collapse. In our experiments proximal collapse after distal microinjection appeared to be due to leakage at the proximal puncture site and did not occur when procedures were taken to minimize leakage i.e. use of pipettes with small tips for micropuncture and/or by leaving the capsule intact. Thus we were unable to obtain results which were confirmatory of Thurau and Schnermann interpretation of their experimental results, and we be

66 % even though the control clearance values were in the low range of normal (re calculated to approximately 0.5 ml/min 100 g b.w.). Fractional reabsorption rose in two rats in which the inulin clearance was reduced by more than 90 %. Although indicating a general relationship between filtration and reabsorptive rates, we do not believe that their results exclude significant increases in fractional reabsorption with reduced inulin clearance. They were not recollection experiments, and the large scatter in their results and the possibilities of technical difficulties in collection of tubular fluid during aortic compression mentioned above may account for the discrepancies between their results and ours.

Gertz *et al.* (1965) found directly measured and estimated proximal fractional reabsorption in normally hydrated nondiuretic rats to increase when arterial pressure was reduced. They concluded that this increase resulted from increased transit time since reabsorptive capacity determined by the split oil droplet method was constant and independent of renal blood flow and Cin. These authors did not relate individual F/P inulin ratios to corresponding Cin values, complicating a direct comparison of their results with ours. It appears, however, that there was no significant increase in F/P inulin ratios until Cin was reduced below 0.5 ml/min 100 g b.w., largely in accordance with our observations.

Brunner, Rector and Seldin (1966) reported that aortic compression had a different effect in unilaterally nephrectomized rats depending upon their pretreatment. In rats given 5—6 ml of isotonic saline during their operative preparation, reduction in arterial pressure to 60—65 mm Hg resulted in an average decrease of 28 % in tubular volume and of 48 % in Cin from a control average of 9.5 ml/min kg b.w., but proximal transit time, halftime for droplet reabsorption and estimated fractional reabsorption were all unchanged. In rats not given saline during their operative preparation, control Cin averaged 5.4 ml/min kg b.w., and a decrease in arterial pressure to 80—85 mm Hg was associated with a 63 % reduction in Cin and increased transit time, halftime for droplet reabsorption and estimated fractional reabsorption. Luminal volume was not significantly reduced. Furthermore, Rector, Brunner and Seldin (1966) found no change in proximal F/P inulin ratios in recollection experiments in 8 tubules of two rats undergoing isotonic saline diuresis when the GFR was reduced by 37 or 52 % from a value of 11.5 and 12.2 ml/min kg b.w., respectively. They concluded from their observations that proximal tubular reabsorption is a function of tubular volume and that glomerulotubular balance is maintained when tubular volume falls in proportion to reduction in GFR. But as Brunner and associates (1966) point out, the proportionality between rate of tubular reabsorption and luminal volume did not always hold for reasons not apparent.

The observations of the Dallas group (1966) in respect to halftime of droplet reabsorption thus did not agree with those of Gertz and co-workers (1965), whereas their transit time measurements agreed when the experimental conditions were similar. The estimates of fractional reabsorption of both groups are in general agreement with our predictions from OT experiments but the mechanism they postulate in explanation of this relationship is totally different.

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Influence of Acetylcholine and Biogenic Amines on Branchial, Pulmonary and Systemic Vascular Resistance in the African Lungfish, Protopterus Aethiopicus

By

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Abstract

JOHANSEN, K. and O. B. REITE. *Influence of acetylcholine and biogenic am nes on branchial pulm nary and syst mic vascular resistance in th African lungfish Protopterus aethiopicus* Acta physiol. scand. 1968. 74 465—471

The effects of acetylcholine and biogenic amines on the main vascular beds in lungfish were studied in intact animals and in isolated preparations perfused with physiological sol tion. Acetylcholine histamine and serotonin increased the branchial as well as the pulmonary and the systemic ascular resistance. The sensitiv ty to acetylcholine was highest in the branchial blood ssels Adrenaline and noradrenaline caused increased resistance in pulmonary and systemic blood vessels, whereas the branchial ascular bed usually esponded by dilatation. The results are discussed in relation to the vascular responses obtained with the same pharmacological agents in other species of fish.

The circulatory system of fish has a branchial and a systemic vascular bed which are coupled in series. In lungfish the post-branchial circulation gives rise to both the pulmonary and the systemic ascular circuits Due to th only partial anatomical separation of these circu ts, resistance hanges in each of the principal vascular beds (branchial pulmonary and systemic) will mutually affect each other Rapid changes in distribution of blood do occu (Johansen, Lenfant and Hanson 1968) but the mechanisms involved re unknown.

The first requirement for evaluation of the role of known hormonal or neurohumoral mediators in the regulation of regional blood flow in lungfish is information about their pharmacological actions in the different sections of the circulatory system. The present investigation wa therefore nitiated to study the effects of acetylcholine and biogenic amines on the arious vascular beds of the African lung fish, *Protopterus aethi picus* as compared to effects previously demonstrated in other species of fish.

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Methods

Ten specimens of the African lungfish, *Prot pt us aethi picus* with body weights of 1.4—6.1 kg were ca ght in Lake Victoria, Uganda, and flow to Oslo, Norway where they were kept in fresh water aquaria at temperature of about 23 C.

Prior to an experiment, fish were prepared under tricaine methane sulfonate (MS 222, Sandoz) anesthesia with chronically implanted intravascular catheters. In all fish, a dorsal aortic catheter was implanted through a branch of the coeliac artery. In some specimens, additional catheters were placed in the pulmonary ein, the ena cava and the ventral aorta, in the latter by retrograde cannulation through the uninterrupted third branchial artery. Transvascular blood pressure gradients could thus be recorded across the branchial, pulmonary and systemic vascular beds. Pressures and pressure gradients were studied during the recovery from anesthesia as w ll as under normal conditions with the fish resting in well aerated ater and the changes which occurred in conjunction with injections of acetylcholine and biogenic amines were observed.

In addition, controlled perfusion of the various vascular beds in freshly killed fish was performed. Perfusion technique was the same as described elsewhere (Reite 1968) using pulsatile constant but controllable olume flow pump. The perfusion fluid consisted of six parts of mammalian Ringer solution (0.86 % N Cl, 0.05 % $CaCl_2$ and 0.03 % KCl) diluted with one part of distilled water. Changes in perfusion pressures reflected active resistance changes i the perfused vascular beds. The aso-active drugs used on i tact fish were also tested in the perfused preparations. In both cases the influence of the corresponding pharmacological blocking agents was also studied. The temperature of the perfusion fluid was the same as the environmental temperature of the fish when alive.

The pressure transducers were Statham Pb 23 Gb and the differential transducer Pb 23 H. The pressures were recorded on Sanborn or Offner dynographs. Drugs used were adrenaline hydrochloride noradrenaline bitartrate histamine phosphate serotonin creatinine sulfate acetylcholine chloride isoproterenol hydrochloride, propranolol hydrochloride, atropine sulfate phentolamine mesylate mepyramine maleate and methysergide maleat. Doses of biogenic amines are expressed as free base for all other drugs the given dose represents the amount of the salt.

Results

Experiments in intact fish

Most of these experiments involved continuous recording of the dorsal aortic blood pressure while injecting drugs i.v. or i.a. Fig 1 shows the typical pattern of the responses to ntravenous injections of various vaso-active amines at dose levels of 1—20 µg. Histamine caused an initial pressure rise, followed by a rapid decline down to or slightly below the pre injection level before return to normal pressure

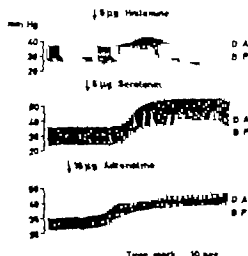


Fig. 1. Changes in dorsal aortic blood pressure (D.A.B.P.) in the Africa lungfish following administration of histamine serotonin and adrenaline into the ena cava.

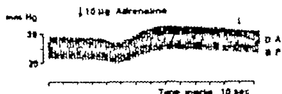


Fig. 2. A transient decrease in dorsal aortic blood pressure which occasionally preceded the pressure rise after intravenous administration of adrenaline is evident in this recording.

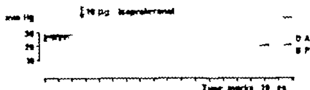


Fig. 3. Blood pressure decrease caused by intravenous injection of isoproterenol.

Adrenaline and serotonin both caused a pressure rise of long duration. In some experiments (an example is given in Fig. 2) adrenaline showed a dichotomous effect when injected into the vena cava, a pressure drop preceding the pressure rise. The latter was always the more prominent. When adrenaline was injected directly into the dorsal aorta, such a transient pressure drop never appeared. The effects of noradrenaline were similar to those of adrenaline. Isoproterenol (10 µg) usually caused a decrease in dorsal aortic blood pressure (Fig. 3). Fig. 4 indicates the response to injections of small doses of acetylcholine (1—2 µg) into the vena cava. Heart rate was unchanged. A markedly reduced pulse pressure in the dorsal aorta was caused by a systolic pressure drop while there was only a moderate reduction in diastolic pressure. To produce a change in heart rate after intravenous injection of acetylcholine, the dose level had to be raised considerably, in most cases above 5 µg, and in some up to 10 µg. Injections of small doses of acetylcholine directly into the post-branchial arterial circulation were either ineffective or caused a small increase in pressure with no change in pulse pressure. However, the dose required to produce the pressure rise was usually high enough to cause a slowing of the heart.

In fish cannulated for measurements of pressure gradients across the branchial and systemic vascular beds, recordings with the differential transducer revealed that acetylcholine injections (1—5 µg) consistently caused an increased pressure gradient across the branchial vascular bed. The same dose levels effected only a small and inconclusive increase in the gradient across the systemic vascular bed. The response to adrenaline and noradrenaline injections was not consistent when measured across the branchial vascular bed. There was either no change or a slightly reduced pres-

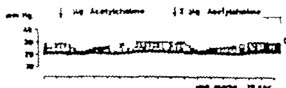


Fig. 4. Effects of small doses of acetylcholine on the blood pressure.

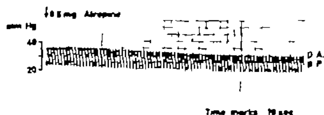


Fig. A marked decrease in blood pressure was consistently observed after atropinization. Counting of heart beats over several one-minute periods before and after injection of atropine revealed no change in the frequency of the heart.

sure gradient. Across the systemic and pulmonary beds, however the catecholamines caused a noticeable increase in the pressure gradient.

Atropine (0.5 mg) reduced dorsal aortic blood pressure without affecting heart rate (Fig. 5) and the effects of subsequent injections of acetylcholine were inhibited. Differential recording in conjunction with atropine injection disclosed a slight reduction in the branchial pressure gradient, but no change in the pressure gradient across the systemic blood vessels. Injection of phentolamine (1 mg) resulted in a decrease in blood pressure and occasionally transient bradycardia, and the pressure response to catecholamines was completely blocked. Propranolol (1 mg) increased dorsal aortic blood pressure.

In all *in vivo* experiments, it was noted that as the animal recovered more completely from the anesthesia, secondary effects of the drug injections appeared. Some drugs (*e.g.* histamine and serotonin) often caused violent behaviour presumably due to central nervous interference. Furthermore, histamine invariably elicited an increase in breathing rate whereas serotonin injections usually caused repetitive expirations.

Perfusion experiments

The perfusions were arranged so that the perfusion pressures of the various vascular beds were in the range of the normally occurring blood pressure. Drugs were dissolved in physiological solution and either injected in small volumes (0.1—1 ml) as single doses into the perfusion circuit, or added to the perfusate reservoir.

Fig. 6 demonstrates the changes in perfusion pressure of the pulmonary vascular bed when acetylcholine and adrenaline respectively were injected into the perfusion circuit. Doses of 2—50 μg were tested, and both drugs showed a marked pressor effect. Similar dose magnitudes of histamine, serotonin and noradrenaline also resulted in a pressure rise. The effects of acetylcholine, histamine serotonin and catecholamines could be abolished by adding respectively atropine (1—5 μg/ml) mepyramine (5—15 μg/ml) methysergide (—4 μg/ml) and phentolamine (3—5 μg/ml) to the perfusion fluid.

The systemic vascular bed showed responses qualitatively similar to those obtained when perfusing the pulmonary circuit.

Perfusion of the branchial vascular bed gave results differing in essential respects from those obtained in the pulmonary and systemic circuits. Injections of single doses (2—50 μg) of adrenaline or noradrenaline at low perfusion pressures usually gave no change in pressure, while similar doses at higher perfusion pressures often

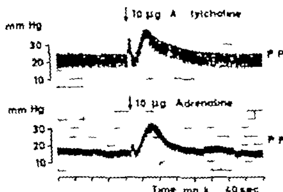


Fig. 6. Recording of perfusion pressure (P P) during artificial perfusion of the pulmonary blood vessels. Both acetylcholine and adrenaline caused marked increase in vascular resistance. The transient pressure rise associated with injection of single doses of the pharmacological agents is produced by the volume of the injected fluid.

resulted in a marked pressure drop. Large doses of adrenaline (50—100 µg) at times caused elevated perfusion pressure. Fig. 7 shows an experiment involving branchial perfusion with acetylcholine-containing perfusion fluid. The upper recording demonstrates a clear pressure rise after acetylcholine (2 µg/ml) has been added to the perfusate. Subsequent injections of adrenaline and noradrenaline successively in single doses (25—100 µg) into the perfusion circuit, now revealed a distinct pressure drop. Propranolol and phentolamine added to the perfusate (2—5 µg/ml) did not block the dilatatory effect of adrenaline and noradrenaline but led to an increase and decrease respectively in the perfusion pressure. Acetylcholine, histamine and serotonin administered in doses of 2—50 µg caused an increase in branchial resistance, and the action of these drugs could be blocked in a similar way as in the pulmonary and systemic vascular beds.

Discussion

The circulatory system of the African lungfish is shown schematically in Fig. 8. The branchial circulation differs in important aspects from branchial circulation in other fish, in that the vascular exchange area represented by the primary and secondary

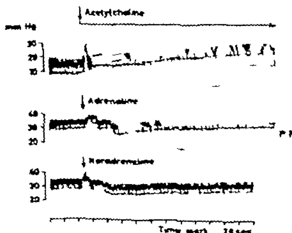


Fig. 7. Perfusion of branchial blood vessels. The upper recording shows the increase in perfusion pressure which occurred when acetylcholine (2 µg/ml) was added to the perfusion fluid. In the two lower recordings adrenaline (100 µg) and noradrenaline (100 µg) were injected as single doses during continued administration of the same concentration of acetylcholine.

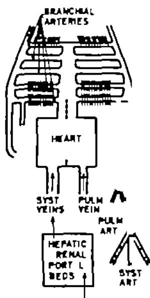


Fig 8 Schematic drawing of the anatomical arrangement of the mal vascular beds in the African lungfish (*Protopterus aethiopicus*)

gill filaments is much reduced. Two aortic arches (III and IV) are completely devoid of gill filaments, with branchial arteries as continuous tubes The blood may take alternate routes, with major flow through the direct channels of arches III and IV or through the gill filaments branching off from the arteries in arches V and VI depending on the relative vascular resistance in the respective parts of the nchial vascular bed. An effective increase in the post branchial perfusion pressure y depend on increased energy released by cardiac contraction, and/or a reduc ion in the vascular resistance across the branchial vascular bed. The relativ flow distribution post branchially will depend on vasomotor changes in the individual sections of the parallel arrangement of systemic and pulmonary vascular beds.

Several investigators have shown that adrenaline usually dilates the blood essels in isolated gill preparations (Krawkow 1913 Keys and Bateman 1932, Östlund and Fänge 1962, Steen and Kruysse 1964) whereas it elevates the blood pres re of fish (MacKay 1931 Lutz and Wyman 1932, Mott 1951 Randall and Stevens 1967 Reite 1968) The study of Reite (1968) shows that both inhibitory and stimulatory vascular actions of adrenaline and noradrenaline in fish can be explained according to the general concept of adrenergic α and β receptors (Ahlquist 1948) Results obtained in the present study indicate that branchial and systemic blood essels in lungfish respond to catecholamines in a way similar to the corresponding blood vessels of other fish Phentolamine and propranolol apparently have direct ascular effects. Considering the high dose levels of adrenaline and noradrenaline required to cause dilatation of the branchial blood essels, the applied concentrations of propranolol were probably inadequate for blockade The decrease in dorsal aortic blood pressure following administration of isoproterenol (Fig. 3) suggest that β-adrenergic receptors may be present somewhere in the post-branchial part of the circulatory system.

It is evident that both histamine and serotonin increase the resistance of all the principal vascular beds in lungfish. The strong vascular response to histamine is unique among fish (Reite 1968)

Branchial vasoconstriction in response to acetylcholine has earlier been demonstrated in the excised gills of teleosts by Östlund and Fänge (1962) In perfusion experiments, Reite (1968) found that acetylcholine produced constriction of systemic as well as branchial blood vessels from both fish and jawless vertebrates, but that large differences in sensitivity may be present among the different vascular beds in a particular species. The experiments now reported reveal that in lungfish the branchial blood vessels are markedly more sensitive to acetylcholine than are systemic and pulmonary vessels, and allow the conclusion that the principal effect of small doses of acetylcholine (1—5 μg) is an increased vascular resistance in the gills. Larger doses (5—10 μg) are needed to produce an effect on systemic and pulmonary vessels or on heart rate.

It has been mentioned above that changes in regional distribution of blood flow between the pulmonary and systemic vascular circuits in lungfish can only result from a change in the relative vascular resistance of these major networks. Knowing that the systemic resistance remains unchanged following administration of atropine, the pressure drop observed in dorsal aorta (Fig. 5) implies a reduced resistance in the pulmonary vascular bed. Whether or not a cholinergic vasoconstrictor tonus prevails in the pulmonary blood vessels of lungfish cannot yet be fully assessed, but the experiment with atropinization suggests that endogenously released acetylcholine may have functional importance. This suggestion is supported by the observation that pulmonary blood flow may double spontaneously with an unchanged total post branchial flow and that such changes usually entail an increased use of the lung in aerial respiration (Johansen *et al.* 1968)

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References

AHLQUIST R. P. A study of the adrenotropic receptors *Amer J Physiol* 1948, *153* 586—600
JOHANSEN K. C LENFANT and D HANSON *Dynamics of circulation in Dipnoi* 1968. (Manuscript in preparation)
KEYS, A. and J B BATEMAN, Branchial responses to adrenaline and pitressin in the eel. *Biol. Bull mar biol Lab Woods Hole* 1932, *63* 327—336
KRAWKOW N P Über die Wirkung von Giften auf die Gefässe isolierter Fischkiemen. *Pflügers Arch ges Physiol* 1913 *151* 583—603
LUTZ B R and L C WYMAN The effect of adrenaline on the blood pressure of the elasmobranch, *Squalus acanthias Biol Bull mar biol Lab Woods Hol* 1932 *62* 17—22.
MACKAY M E The action of some hormones and hormone-like substances on the circulation in the skate. *Cont Can Biol* N S 1931 *7* 19—29
MOTT J C Some factors affecting the blood circulation in the common eel (*Anguilla anguilla*) *J Physiol* (Lond.) 1951 *114* 387—398
ÖSTLUND E and R FÄNGE, Vasodilation by adrenaline and noradrenaline and the effects of some other substances on perfused fish gills *Comp Biochem Physiol* 1962, *5* 307—309
RANDALL, D J and E DON STEVENS, The role of adrenergic receptors in cardiovascular changes associated with exercise in salmon *Comp Biochem Physiol* 1967 *1* 415—4 4
REITE O B The evolution of vascular smooth muscle responses histamine and 5-hydroxy tryptamine I Occurrence of stimulatory actions in fish. *Acta physiol scand* 1969 *I* press
STEEN J B and A KRUYSSE The respiratory function of teleostean gills. *Comp Biochem Physiol* 1964 *12* 127—142.

Acta physiol. scand. 1968. 74. 472—479

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Potentiation by Smooth Muscle Stimulants of the Hypogastric Nerve — Vas Deferens Preparation from Normal and Castrated Guinea Pigs

By

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Abstract

SJÖSTRAND, N. O. and G. SWEDIN. *Potentiation by smooth muscle stimulants of the hypogastric nerve-vas deferens preparation from normal and castrated guinea-pigs.* Acta physiol. scand. 74. 472—479.

It is shown that a variety of smooth muscle stimulants cause marked potentiation of the motor response of the isolated vas deferens to hypogastric nerve stimulation. The substances used were adrenaline, noradrenaline, acetylcholine, histamine, serotonin, angiotensin, bradykinin, Substance P and barium chloride. The doses producing this potentiation were in most cases 10—1000 times lower than those necessary to cause a direct contraction of the vas deferens. There were no clearcut differences between castrated and normal animals. Oxytocin and vasopressin were without effect while different prostaglandins (PGE, PGF and a crude PG preparation) caused inconsistent responses.

Possible mechanisms for the potentiation are discussed and it is suggested that the most probable explanation is a direct effect on the smooth muscle, but an effect on the transmitter stores of the postganglionic adrenergic neuron cannot be ruled out. Possible functional implications are further discussed.

There is evidence in the literature that smooth muscle stimulants may potentiate the motor response of plain muscle to adrenergic nerve stimulation. Thus circulating noradrenaline increases the responses of certain vascular beds to sympathetic stimulation (Burn and Rand 1960). Angiotensin (Zimmerman and Gomez 1965, Zimmerman 1967) and vasopressin (Hertting and Suko 1966, Bernhard *et al.* 1968) have also been found to produce similar effects. Furthermore Substance P (Beleslin *et al.* 1960) and serotonin (Bacq and Renson 1961) enhance the response of the cat nictitating membrane to sympathetic nerve stimulation.

In a previous communication (Sjöstrand 1961) it was reported that several biogenic amines caused a considerable potentiation of the response of the guinea-pig vas deferens to hypogastric nerve stimulation. The present investigation was undertaken in order to further investigate this potentiation to different amines and to extend the study also to other wellknown smooth muscle stimulants.

Material and methods

Vasa deferentia from 50 normal and 8 castrated male guinea-pigs with an average weight of 500 g were used. The castration was performed 10—110 days before the experiments.

The vas deferens with the hypogastric nerve was prepared according to the method of Hukovic (1961). The organ was placed in 40 ml (in some expts 50 ml) bath containing Tyrode solution at 37 C and aerated with 6.5 % CO_2 in O_2. The contractions were recorded isotonically on a smoked drum with frontal lever (magnification 16 1). The hypogastric nerve was placed on stimulating electrodes (2 mm between the two platinum electrodes). In one series of experiments the nerve and electrodes were immersed in liquid paraffin. In the others the nerves and electrodes were kept in the solution of the bath. The nerve was stimulated every one or two min for 5 sec with supramaximal voltage (7—15 V when mounted in paraffin oil, 50—100 V if the nerve was in the Tyrode solution) and duration of 2 msec, the frequency being in the range of 5—70 per sec. The electrodes were placed 0.5—4 cm from the organ. Every preparation could be used for at least 5—6 hrs.

The following substances were used: adrenaline and noradrenaline as hydrochloride or bitartrate, 5-hydroxytryptamine (serotonin) as creatine sulphate, acetylcholine chloride or bromide, histamine dihydrochloride, prostaglandins (PGE, PGF and crude preparation of PG (Eliasson 1959)), Substance P (in a preparation according to Euler), hypertensin, oxytocin (Syntocinon® Sandoz), vasopressin (Sandoz), bradykinin (Sandoz) and barium chloride. Oxytocin, vasopressin and bradykinin were given in the commercial solvents, the other drugs in aqueous solutions.

The following blockers were used: Atropine sulphate, 2-bromlysergic acid diethylamide (BOL, Sandoz), phentolamine chloride (Regitin® Ciba), phenbenzamine chloride (Lergitin® Recip). The Tyrode solution had the following composition: 0.8 % NaCl, 0.02 % KCl, 0.02 % $CaCl_2$, 0.01 % $MgCl_2$, 0.1 % $NaHCO_3$, 0.005 % $NaH_2PO_4 \cdot H_2O$ and 0.1 % glucose.

Results

Biogenic amines

The vas deferens was contracted directly by addition to the bath of adrenaline (A), noradrenaline (NA), acetylcholine (ACh), histamine and serotonin (5-HT) (Fig. 1 B). However large concentrations had to be used (10 —10 M) to elicit a clearcut response. Because of rather great differences in sensitivity of different preparations a definite numerical comparison is hard to present.

The biogenic amines in 10—1000 times lower concentrations (2 × 10 —4 × 10 M) than those necessary to elicit direct contraction caused conspicuous potentiation of the organ to nerve stimulation. In general the amines were ranked in the following order: ACh > A ≈ NA > histamine > 5-HT (Fig. 1 A). The potentiation seen was often very longlasting (up to 1 hr, Fig. 2) but disappeared almost immediately after washing (Fig. 1 A). Changes of frequency (Fig. 3) and duration (Fig. 4) of stimulation did not alter the ratio between initial and potentiated contractions. The potentiation was of the same magnitude with the stimulating electrode on different positions on the hypogastric nerve (0.5—4 cm from the organ).

There were no apparent differences in responses of organs from *castrated* guinea pigs when compared to those of controls.

Blockers. *Atropine* in small doses (0.5—5 μg in the bath) abolished the potentiation due to ACh and sometimes that of 5-HT. The effects of the catecholamines and histamine were left unimpaired.

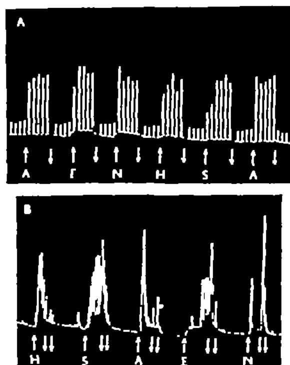


Fig. 1 Comparison of the effects of biogenic amines on the guinea-pig vas deferens
A Contractions of the isolated vas deferens in response to nerve stimulation of supramaximal voltage (80 V msec, 7 pulses per sec for 5 sec at 1 min intervals) A = acetylcholine 0.1 × 10 M, E = adrenaline 1 × 10⁻⁶ M, N = noradrenaline 1 × 10⁻⁶ M, H = histamine 2.4 × 10 M, S = serotonin 4 × 10⁻⁶ M.
B. Isolated vas deferens (same preparation as in A) H = histamine 0.4 × 10⁻⁶ M, S = serotonin 1 × 10⁻⁶ M, A = acetylcholine 0.1 × 10⁻⁶ M, E = adrenaline 1 × 10 M, N = noradrenaline 0.6 × 10 M.

Ph tol m 10—50 μg blocked the potentiation caused by the catecholamines to a lesser extent than of 5-HT while it had no effect on the response to ACh histamine

Phenben.amine 5—50 μg) obliterated the effect of histamine and reduced that of ACh. The potentiation caused by A and NA was unaffected.

BOL 10—50 μg inhibited the effect of 5-HT and also that of A and NA but not that of the other substances

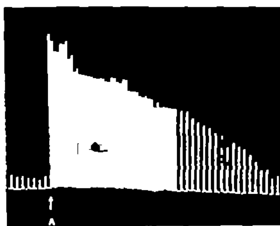


Fig. 2. Contractions of the isolated guinea-pig vas deferens in response to nerve stimulation of supramaximal voltage (80 V 2 msec, 5 pulses per sec for 5 sec at 1 min intervals) A = acetylcholine 2 × 10 M.

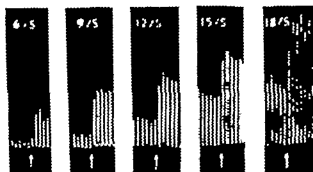


Fig. 3 Contractions of the isolated guinea-pig as deferens in response to nerve stimulation of supramaximal oltage (90 V 2 msec for 5 sec at 1 min inter als) The guinea-pig was castrated 60 days before the ex periment. Stimulation frequency indicated by numbers above contractions. At arrow acetylcholine 2×10 M

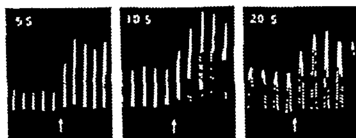


Fig. 4. Contractions of the isolated guinea-pig as deferens to nerv stimulation of supramaximal oltage (90 V 2 msec, 10 pulses per sec at 1 min intervals) Duration of stimulation indicated by numbers abov contractions At arrow acetylcholine 3×10^{-9} M.

Other mooth muscle t mulants

Barium klorid (0 1—0.5 mg in the bath) exerted a marked potentiation on the vas deferens preparation (Fig. 5 A)

Distinct effects were also obtained with *b adykinin* (20—50 *ng*, Figs. 5 B 6 C and D) *Substan P* (20—60 Units, Fig 5 C) and *angiotensin* (20—50 *µg* Fig 6 A and B)

A moderate decrease in potentiation to angiotensin was often seen when th electrodes were placed close to the organs (0.5 cm, Fig. 6 A and B This wa not the case with the other substances used (.g bradykinin, Fig 6 C and D) At this *timulation point hexamethonium (300 µg) did not block the response to hypo*gastric nerve umulation (*f* Sjöstrand 1965 and Ferry 196)

The effects of the *p o tagl nd ns* were inconsistent Usuall PGE (0 1—50 *µg*) PGF (0 1—10 *µg*) and the crude PG (0 1—50 Unit caused a slight nhibition of the vas deferens but n a few expts a moderate potentiation could be seen In some cases no effect at all was obtained No certain correl tion between gi en dose and kind of response was observed

Oxytocin (0 01—0 Units and *a p n* 0 01 -0 Units w re w thout effect (In some expts an inhibition was obser ed This was evidently due to an effect of phenylbutol in th solvent sinc t ould be produced by this substance alone.)



Fig. 5. Contractions of the isolated guinea-pig vas deferens to nerve stimulation of supramaximal voltage (15 V, 2 msec, 7 pulses per sec for 5 sec at 2 min intervals). Bath volume 50 ml.
A. BA = 0.5 mg $BaCl_2$.
B. BR = 20 µg bradykinin.
C. SP = 50 Units Substance P

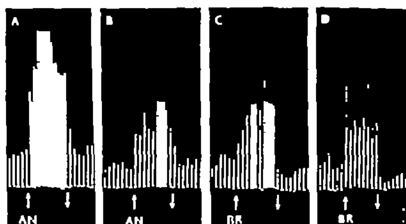


Fig. 6. Contractions of the isolated guinea-pig vas deferens in response to nerve stimulation of supramaximal voltage (70 V, msec for 5 sec, 1 min intervals). Bath volume 40 ml.
A. Electrodes about 4 cm from the vas deferens. Stimulation frequency 5 per sec. AN = 50 µg angiotensin.
B. Electrodes about 0.5 cm from the vas deferens. Stimulation frequency 4 per sec. AN = 50 µg angiotensin.
C. Electrodes about 4 cm from the vas deferens. Stimulation frequency 5 per sec. BR = 50 µg bradykinin.
D. Electrodes about 0.5 cm from the vas deferens. Stimulation frequency 4 per sec. BR = 50 µg bradykinin.

Discussion

In the present study it has been shown that a variety of smooth muscle stimulants causes a marked potentiation of the motor response of the isolated vas deferens to hypogastric nerve stimulation. The doses producing this potentiation are in most cases 10—1000 times lower than those causing a direct contraction of the vas deferens. There are no clearcut differences between organs from castrated and

control animals. From the present study no certain conclusion can be drawn about the effects of the prostaglandins. The lack of effect of oxytocin, vasopressin and castration is noticeable.

Potentiation of the vas deferens to nerve stimulation has previously been reported for adrenaline, noradrenaline and acetylcholine by Sjöstrand (1961) and Holman and Jowett (1964). The same effect was found by Sjöstrand (1961) to Substance P, Benelli *et al.* (1964) to angiotensin and Mantegazza and Naimzada (1965) to PGE. No detailed discussion on the mechanism of this potentiation has, however, been presented.

The possible sites of the action of the different smooth muscle stimulants are at 1) the peripheral ganglionic synapse, 2) the postganglionic nerve terminal and 3) directly on the smooth muscle.

Evidence has been presented for the existence of a peripheral ganglionic synapse located near the guinea pig vas deferens and belonging to the sympathetic innervation of this organ (Sjöstrand 1962 a, b, Bentley and Sabine 1963, Birmingham and Wilson 1963, Ferry 1963 and others, *cf.* Sjöstrand 1965 and Ferry 1967).

Previous investigations have indicated that, besides acetylcholine, histamine (Trendelenburg 1954), serotonin (Trendelenburg 1957), Substance P (Beleslin *et al.* 1960), bradykinin and angiotensin (Lewis and Reit 1965) and barium (Douglas *et al.* 1961) may stimulate and facilitate the transmission through the superior cervical ganglion. Catecholamines in low doses may also potentiate transmission through this ganglion (Bülbring and Burn 1942, Konzett 1950, Trendelenburg 1965) although most investigators have found a depressant action of catecholamines on ganglionic transmission (Marrazzi 1939 and others, *cf.* Norberg and Sjöqvist 1966). It seems, however, not probable that facilitation of ganglionic transmission is the major cause of the potentiation seen in the present study, because it is seen also with the stimulating electrodes close (about 0.5 cm) to the vas deferens. In this case the stimulation is mainly postganglionic (*cf.* Sjöstrand 1965, Ferry 1967). Furthermore, the inhibition by atropine of the acetylcholine induced potentiation speaks against a ganglionic site of action of this substance. In the case of angiotensin an effect on the ganglionic transmission may contribute to the potentiation, since the effect was slightly reduced when the stimulation was applicated near vas deferens (*cf.* Fig. 6).

Another possible explanation of the observed potentiation could be an increase of the amount of transmitter released per stimulus from the adrenergic nerve terminals. It is well known that serotonin can stimulate the autonomic postsynaptic axon (Rocha e Silva *et al.* 1953, Gaddum and Picarelli 195). It has also been suggested that angiotensin may have a direct action on the transmitter store of the postganglionic nerve terminal (Robertson and Rubin 1962, Benelli *et al.* 1964). Hertting and Suko (1966) and Hughes (1968) denies such an action. In this connection it should be mentioned that Hughes (1968) in contrast to the present finding and that of Benelli *et al.* (1964) found no potentiation of the vas deferens preparation to angiotensin.

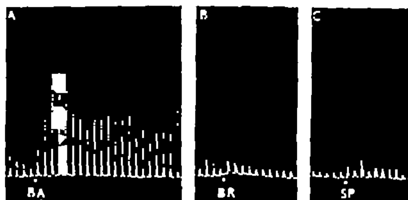


Fig. 5. Contractions of the isolated guinea-pig vas deferens to nerve stimulation of supramaximal voltage (15 V, 2 msec, 7 pulses per sec for 5 sec at 2 min intervals). Bath volume 50 ml.
A. BA = 0.5 mg $BaCl_2$.
B. BR = 20 µg bradykinin.
C. SP = 50 Units Substance P

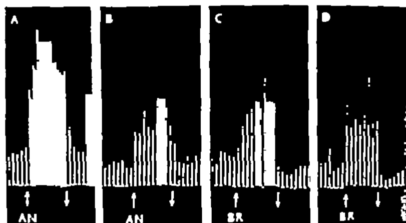


Fig. 6. Contractions of the isolated guinea-pig vas deferens in response to nerve stimulation of supramaximal voltage (70 V, msec for 5 sec at 1 min intervals). Bath volume 40 ml.
A. Electrodes about 4 cm from the vas deferens. Stimulation frequency 5 per sec. AN = 30 µg angiotensin.
B. Electrodes about 0.5 cm from the vas deferens. Stimulation frequency 4 per sec. AN = 30 µg angiotensin.
C. Electrodes about 4 cm from the vas deferens. Stimulation frequency 5 per sec. BR = 30 µg bradykinin.
D. Electrodes about 0.5 cm from the vas deferens. Stimulation frequency 4 per sec. BR = 30 µg bradykinin.

Discussion

In the present study it has been shown that a variety of smooth muscle stimulants causes a marked potentiation of the motor response of the isolated vas deferens to hypogastric nerve stimulation. The doses producing this potentiation are in most cases 10—1000 times lower than those causing a direct contraction of the vas deferens. There are no clearcut differences between organs from castrated and

BIRMINGHAM A. T and A. B. WILSON Preganglionic and postganglionic stimulation of the guinea-pig isolated vas deferens preparation. *Brit J Pharmacol* 1963 *21* 569—580.
BÜLBRING, E. and J H. BURN An action of adrenaline on transmission in sympathetic ganglia which may play part in shock. *J Physiol. (Lond.)* 194_ *101* 289—303
BURN, J H. and M. J RAND The relation of circulating noradrenaline to the effect of sympathetic stimulation. *J Physiol. (Lond)* 1960. *150* 295—305.
DOUGLAS, W W D W LYWOOD and R. W STRAUB The stimulant effect of barium on the release of acetylcholine from the superior cervical ganglion. *J Physiol. (Lond)* 1961 *156* 515—522.
ELIASSON, R., Studies on prostaglandin. *Act physiol. sc d* 1959 *46*. Suppl. 158.
FERRY, C. B., The innervation of the vas deferens of the guinea-pig. *J Physiol. (Lo d)* 1963 *166* 16 P
FERRY C. B., The innerv tion of the vas deferens of the guinea-pig. *J Physiol. (Lond)* 1967 *192* 463—478.
GADDUM, J H. and Z. P PICARELLI, Tw kinds of tryptamine receptor *Brit J Pharmacol.* 1957 *12* 323—328.
HERTTING, G. and J SUKO Influence of angiotensin, vasopressin or changes in flow rate on vaso-constriction, changes in volume and (H)-noradrenaline release *Brit J Pharmacol.* 1966. *26* 686—696.
HOLMAN, M. and A. JOWETT Some actions of catecholamines on the smooth muscle of the guinea-pig vas deferens. *Aust J exp Biol. med Sci.* 1964 *42* 40—53
HUGHES, L E An investigation of the effects of angiotensin on the release of neurohumoral transmitters at motor adrenergic and cholinergic nerv terminals. *J Pharm. Pharmac* 1968. *20* 116—124
HUKOVIĆ, S., Responses of the guinea-pig as deferens. *Brit J Pharmacol* 1961 *16* 188—224
KONZETT, H., Sympathicomimetica und sympathicolytica am isoliert durchströmten Ganglion cervicale superior der Katze. *H Ir physiol. pharmacol. Act* 1950 *8* 245—258.
LEWIS, G. P and E. REIT The action of angiotensin and bradykinin on the superior cervical ganglion of the cat. *J Physiol. (Lond.)* 1965. *179* 538—553
MA TROIANNI, P and M. KASIM NASIRALDIN, Attività della prostaglandina E sul preparato nerv ipogastrico-deferente di arie specie animali. *Att A ad med lombarda* 1965 *20* 58—64.
MARRAZZI, A. S., Adrenergic inhibition at sympathetic synapses. *Ame J Physiol* 1939 *127* 738—744
NORBERG, K. A. and F SJÖQVIST New possibilities for adrenergic modulation of ganglionic transmission. *Pharmacol. Rev* 1966. *18* 743—751
ROBERTSON P A. and D RUBIN Stimulation of intestinal nervous lements by angiotensin *Brit. J Pharmacol.* 1962. *19* 5—12
ROCH SILVA, M J R. VALLE and Z P PICARELLI, Pharmacological analysis of the mode of action of serotonin upon the guinea-pig ileum. *Brit J Pharmacol* 1953 *8* 378—388
SJÖSTRAND N O Effect of some smooth muscle stimulants on the motor response of the isolated guinea-pig as deferens to hypogastric nerv stimulation. Nat (Lond 1961 *192* 1190—1191
SJÖSTRAND N O Inhibition by ganglionic blocking agents of the motor response of the isolated guinea-pig vas deferens to hypogastric nerve stimulation *Act physiol. sc d* 1962 a. *54* 306—315.
SJÖSTRAND N O Effect of reserpine and hypogastric denervation on the noradrenaline content of the vas deferens of the guinea-pig *Act physiol sc nd* 1962 b *56* 376—380
SJÖSTRAND N O The adrenergic innervation of the as deferens and the accessory male genital glands. *Act physiol. scand* 1965 *65* Suppl. 257
TRENDELENBURG, U The action of histamine and pilocarpine on the superior ervical ganglion and the adrenal glands of the cat. *Brit J Pharmacol* 1954 *9* 481—487
TRENDELENBURG U Modification of transmission through the superior cervical ganglion of the cat. *J Physiol. (Lond)* 1956 *132* 529—541
TRENDELENBURG, U The action of morphine on the superior ervical ganglion and on the nictitating membrane of the cat. *Brit J Pha macol* 195 *1* 9 -85
ZIMMERMANN B G E aluation of peripheral and central components of action of angiotensin on the sympathetic nervous system. *J Pharmacol p Ther* 196 *158* 1—10
ZIMMERMAN B. G and J GOMEZ, Increased response to sympathetic stimulation in the cutaneous asculature in presence of angiotensin *I J N pharmacol* 1965 *4* 185—193.

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Biliary Bile Acids and Hepatic Ultra-Structure in Hamsters Fed Gallstone-inducing and -dissolving Diets

By

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Abstract

BERGMAN, F., W. VAN DER LINDEN and J. SJÖVALL. *Biliary bile acids and hepatic ultra structure in hamsters fed gallstone-inducing and -dissolving diets.* Acta physiol. scand. 1968. 74. 480—491.

Bile acids in gallbladder and fistula bile and the hepatic structure were studied in hamsters fed gallstone inducing diets with and without supplementation of cholestyramine or thyroxine. The predominant bile acids identified were cholic, chenodeoxycholic, deoxycholic, 7-keto-deoxycholic and 7α,12α-dihydroxy-3-keto-5β-cholanoic acids. Small amounts of 7-ketolithocholic, 12-ketolithocholic, 12α-hydroxy-3-keto-5β-cholanoic, allocholic and (tentatively) ursodeoxycholic acids were also found. The mean of the chenodeoxycholic/cholic acid ratios in hamsters fed a diet favouring the formation of cholesterol gallstones was 0.88 and fell to about 0.04 when 5 % cholestyramine was added to the diet. Thyroxine caused a similar but less striking fall of this ratio. The bile acid/cholesterol ratio was lower in animals on the cholesterol stone inducing diet and was increased by cholestyramine administration which prevented the formation of cholesterol stones. As in previous work, morpho-histochemical changes were found in the hepatic parenchymal cells almost exclusively of hamsters treated with the thyroxine containing diet which induces predominantly pigmented stones. Electron microscopy showed the cells to contain numerous lipid droplets and clusters of autophagic vacuoles. Possible relationships between biliary bile acids, cholesterol and gallstone formation and the effects of cholestyramine and thyroxine are discussed.

In their studies on diet induced gallstone formation, Dam and coworkers have determined the concentrations of conjugated cholic, chenodeoxycholic and deoxycholic acids in the bile of hamsters fed different diets. They found that the ratio of glycine conjugated to taurine conjugated bile acids was influenced by the diet but consistent differences in the ratio of trihydroxy to dihydroxy bile acids were not observed (see review by Dam 1965). Detailed studies on the possible occurrence of bile acids other than those mentioned were not made. In a previous investigation it was shown that oral administration of cholestyramine to guinea pigs fed a deficient diet resulted in the formation of cholesterol stones (Schoenfield and Sjövall 1966 a). The bile acid composition in guinea pigs fed this diet was quite different from that of animals given a commercial chow diet. In hamsters, however, administration of

cholestyramine causes the dissolution of cholesterol stones (Bergman and van der Linden 1966 b 1967) It was therefore of interest to study the bile acid patterns in hamsters fed a gallstone inducing diet with and without cholestyramine. We further aimed at studying the bile acids in hamsters fed a thyroxine containing diet which previous investigations have shown to induce pigmented stones (Bergman and van der Linden 1965 1966 a) An account is also given of the microscopical changes of the liver found in hamsters fed different gallstone inducing and -dissol ving diets.

Experimental

Exp 1 Young hamsters of both sexes, weighing 55—63 g t the start of the experiment were used. All animals were individually caged nd had free access to food nd water By random separation groups comprising 20 hamsters were formed which were fed different diets during period of one month. One diet (N) was given t group of only 10 animals. The following diets were used.

1) N — Normal commercial chow[1]
2) 295. — See Table I
3) 295 Ch. — Diet 295 supplemented with 3 % cholestyramine[2]
4) HK. — Diet 295 with one third of the sucrose replaced by rice starch.
5) HL. — Diet HK supplemented with -thyroxine, 5 mg/100 g food[3]

TABLE I. Composition of diet 295

Sucrose	72.3
Casein (crude)	20.0
Salt mixture (U.S.P XIII No. 2)	5.0
Vitamin mixture	0.5
Choline chloride	0.2
Lard	2.0

After one month on these diets laparotomy was performed under ether narcosis. Gallbladder bile was obtained by direct puncture of the gallbladder A soft polyethylene catheter was then inserted into the gallbladder in the direction of the cystic duct. A ligature was placed around the gallbladder and the tube to hold the catheter in place The common duct was ligated and the polyethylene tube was passed under the skin from the abdominal incision to the back. The tube was taken out through hole in the skin of the neck and was then placed in small ampoule which was fixed to the skin and kept in place with piece of adhesive tape (Fig. 1) Bile was collected after 2 and after 12 hours. Histological examination of liver and thyroid gland were performed as described in previous communications (Bergman & van der Linden 1965, 1966)

Exp Hamsters of t least 5 months and w h minimum eight of 80 g were used. These animals had been fed normal chow diet supplemented ith carrots and sallad. A biliary fistula was created after the common duct had been ligated. Bile w sampled with regular intervals.

Exp 3 Young hamsters of both sexes, weighing 55—63 g ere fed the diets described in experiment 1 during one month. The animals were randomly separated and individually caged. After one month on the different diets they were sacrificed Frozen and paraffin-embedded sections of the liver were prepared for histological examination. I addition 5 animals in each group were used for the electron microscopical part of the study Small tissue samples, not exceeding 1 mm in thickness, from the liver ere fixed in 1 pe cent osmium tetroxide solution, dehydrated and embedded in Epon Luf 1961 Sections were co th

[1] Obtained from Harald Fors AB Holmsund S eden

[2] Generously supplied by Merck, Sharp & Dohme through Erik Lindblom AB Stockholm, Sweden.

[3] Generously supplied by Nyegaard & Co A S Oslo Norway

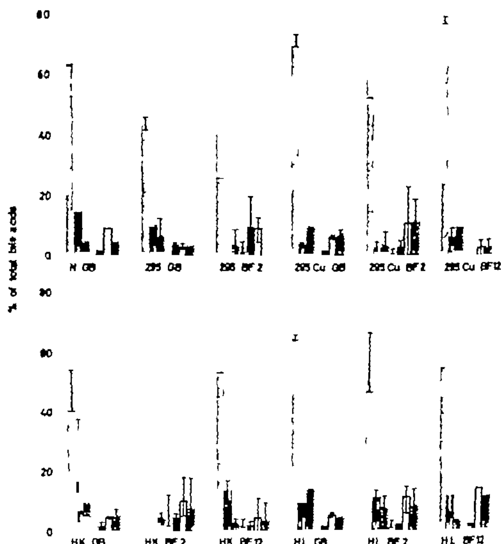


Fig. 3 Percentage composition (g individual bile acid/100 g total bile acids) of bile acids in bile. Vertical lines indicate range of values GB, BF2 BF12 and diet abbreviation see text and Fig. 1

■ — cholic + allocholic acids
▥ — chenodeoxycholic + ursodeoxycholic acids
▢ — deoxycholic acid
— lithocholic acid
▣ — 3α-hydroxy-7-keto- + 3α-hydroxy-12-keto- + 12α-hydroxy-3-keto-5β-cholanoic acids
▢ — 3α,12-dihydroxy-7-keto-5β-cholanoic acid
▢ — 7α,12α-dihydroxy-3-keto-5β-cholanoic acid.

chromatography permitted the identification of the following bile acids (systematic name of the bile acid and RRT of its methyl ester on QF 1 column given in parenthesis) lithocholic (3α-hydroxy-5β-cholanoic acid, 0.53) deoxycholic (3α 12α-dihydroxy-5β-cholanoic acid, 1.00) chenodeoxycholic, (3α,7α-dihydroxy-5β-cholanoic

acid, 1.14), 12-ketolithocholic (3α-hydroxy-12-keto-5α-cholanoic acid, 1.51), 7-ketolithocholic (3α-hydroxy-7-keto-5β-cholanoic acid, 1.71), 12α-hydroxy-3-keto-5β-cholanoic (1.81), cholic (3α,7α,12α-trihydroxy-5β-cholanoic acid, 2.16), allocholic (3α,7α,12α-trihydroxy-5α-cholanoic acid, 2.42), 7-ketodeoxycholic (3α,12α-dihydroxy-7-keto-5β-cholanoic acid, 3.10) and 7α,12α-dihydroxy-3-keto-5β-cholanoic (4.10) acids. Ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid, 1.30) was tentatively identified and the compound with an RRT of 2.65 could not be identified.

The concentrations of total bile acids in bile are shown in Fig. 2. The values for gallbladder bile were similar in the 5 groups of animals; there was possibly a lower bile acid concentration in groups HK and HL. Calculated as a percentage of total solids the bile acid values were slightly lower on diet 295 than on the same diet supplemented with cholestyramine. When an external biliary fistula was created, the concentration of bile acids fell during the first 12 hrs. No difference was observed between the different diet groups.

Fig. 3 shows the composition of the bile acids in the various groups, the amount of the different bile acids being given as a percentage of the total amount. It should be pointed out that the analyses of gallbladder bile, the results of which are given in Fig. 2 and 3, were made on pooled bile samples where at least 4 animals contributed to each separate sample. The minor monohydroxy-monoketocholanoic acids have been grouped together and ursodeoxycholic acid, if present, has been added to chenodeoxycholic acid. Because of difficulties in the separate quantitation of allocholic acid this compound has been included in the percentage of cholic acid. In a few samples allocholic acid appeared to be present in an amount corresponding to 10 per cent of the cholic acid but usually the amount was much smaller.

Comparison of the gallbladder bile of hamsters fed the lithogenic diet 295 with and without cholestyramine (295 CB and 295 Cu GB) revealed a striking difference. In the gallbladder bile of the hamsters fed diet 295 the ratio between chenodeoxycholic and cholic acids ranged from 0.97—0.70 whereas values of 0.03 and 0.05 were obtained in animals on the same diet supplemented with cholestyramine. On diet HK, which is less strongly gallstone provoking this ratio was lower than on diet 295; on normal chow it was still lower. A similar difference appeared when the corresponding fistula bile values were compared. These differences are so large and so constant, while so many animals contributed to the samples analysed that, although their exact statistical significance is difficult to calculate, they can hardly be due to chance.

On diet 295 the chenodeoxycholic acid/cholic acid ratio was less in fistula than in gallbladder bile, the mean values being 0.88 and 0.7, respectively. A similar fall of this ratio after the preparation of a biliary fistula was seen in the hamsters fed the HK diet. The mean value of the chenodeoxycholic acid/cholic acid ratio was 0.66 in the gallbladder bile, 0.44 in the fistula bile obtained between 0 and 2 hrs and 0.19 in the 2—1 hr sample. A fall of this ratio occurred neither in the group fed the 295 Cu diet nor in that reared on diet HL. The proportion of chenodeoxy-

TABLE II. Concentration of cholesterol and ratio of bile acid to cholesterol concentration in gallbladder and fistula bile of hamsters fed different diets

Diet[a]	Sample[a]	Number of analyses[b]	Cholesterol concentration (mg/100 ml)		Bile acid/cholesterol ratio	
			Mean	Range	Mean	Range
N	GB	1	50.5	—	52.9	—
295	GB	3	77.9	48 5—155 0	58.1	24.2— 51.9
295	BF2	3	18.6	14 4— 23.8	18.1	7.5— 29.4
295Cu	GB	2	25.8	24 8 26.9	99 1	79 4 119 2
295Cu	BF2	4	22.2	8.0— 38 0	38.1	17 4— 90.5
295Cu	BF12	1	7.9	—	23 0	—
HK	GB	2	91.5	54 1— 128.9	28.1	16 6 39 6
HK	BF2	3	39.5	35.9— 43.2	17 4	7.8— 28.0
HK	BF12	3	8.4	5.4— 11 7	17 6	6.1— 27.9
HL	GB	2	55.1	54.8 55 4	34.8	33.5 37.0
HL	BF2	3	34 4	22 4— 39.8	13.2	12.3— 15.2
HL	BF12	1	22 9	—	11 7	—

[a] For abbreviations see text and Fig. 2

[b] Analyses of gallbladder bile (GB) were made on pooled samples representing 4—6 animals each. Analyses of fistula bile (BF2, BF12) were made on samples from individual animals.

cholic acid was somewhat lower in hamsters fed diet HL than in those reared on diet HK.

The values for biliary cholesterol concentration showed large individual variations. This was true particularly for the fistula bile. The results of the analyses are summarized in Table II It is seen that the cholesterol concentration in fistula bile was lower than that in gallbladder bile The ratio between the bile acid and cholesterol concentrations was also lower in fistula bile than in gallbladder bile. The highest cholesterol concentrations and the lowest bile acid/cholesterol ratios were found in the hamsters fed diets 295 and HK. The animals given diet 295 supplemented with cholestyramine had a lower biliary cholesterol concentration and a higher bile acid/cholesterol ratio than those fed the unsupplemented diet. The statistical significance of these differences is difficult to evaluate but it should be noted that the differences were found both in gallbladder and fistula bile

Exp 2. After the creation of an external biliary fistula most animals died within 24 hrs. Only 3 animals lived for a longer time with a functioning fistula (26, 66 and 42 hrs, respectively) The results of the bile acid analyses are given in Table III After an initial, marked decrease during the first 8—18 hrs the bile acid concentration increased markedly in two hamsters. This increase was not seen in animal no 3 which was comatose when the 42 hrs sample was collected

Exp 3 Light microscopy The microscopical changes of the liver were in ac

TABLE III Concentration of bile acids in fistula bile collected at different times after operation

Animal	Time after operation (hrs)	Total bile acid concentration* (mg/100 ml)	Bile acids, per cent of total solids*
No. 1	0 — 2.5	809	12.6
	2.5 — 8	39	0.9
	8 — 20	136	4 0
No. 2	0 — 6	411	20.0
	6 — 18	41	3 0
	18 — 30	85	3 4
	30 — 42	124	7 4
	42 — 54	119	5.6
	54 — 66	76	3.3
No. 3	0 — 6	537	22.8
	6 — 18	77	4.9
	18 — 42	49	3 4

Expressed as free bile acids.

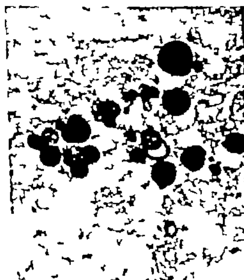


Fig 4



Fig. 5

Fig. 4 Detail of liver cell containing vacuolated dense bodies of variable size × 45 000
Fig. 5. Dense bodies showing characteristic myelin figures. × 150,000

cordance with those found in previous studies (Bergman and van der Linden 1965 1966 a) The thyroxine treated hamsters showed marked fatty degeneration of the liver These changes were less pronounced in animals subsequently fed normal chow and in those fed the unsupplemented diet 295 whereas they had disappeared completely in animals fed this latter diet supplemented with cholestyramine.

Electron microscopy After 2 weeks treatment with the diet containing thyroxine

TABLE II Concentration of cholesterol and ratio of bile acid t cholesterol concentration in gallbladder and fistula bile of hamsters fed different diets

Diet[a]	Sample[a]	Number of analyses[b]	Cholesterol concentration (mg/100 ml)		Bile acid/cholesterol ratio	
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295	GB	3	77.9	48.5—135 0	38.1	24.2— 31.9
295	BF2	3	18 6	14 4— 23.8	18.1	7.5— 29 4
295Cu	GB	2	25.8	24.8 26.9	99 1	79 4 119.2
295Cu	BF2	4	22.2	8 0— 38.0	38.1	17 4— 90.5
295Cu	BF12	1	7.9	—	23.0	—
HK	GB	2	91.5	54 1 128.9	28 1	16.6 39 6
HK	BF2	3	39.5	35.9— 43.2	17 4	7.8— 28.0
HK	BF12	5	8.4	5.4— 11 7	17 6	6.1— 27.9
HL	GB	2	55 1	54.8 55.4	34.8	33.5; 37.0
HL	BF2	3	34 4	22 4— 39.8	13.2	12.3— 15.2
HL	BF12	1	22 9	—	11 7	—

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	30 — 42	124	7.4
	42 — 54	119	5.6
	54 — 66	76	3.3
No. 3	0 — 6	337	22.8
	6 — 18	77	4.9
	18 — 42	49	3.4

Expressed as free bile acids

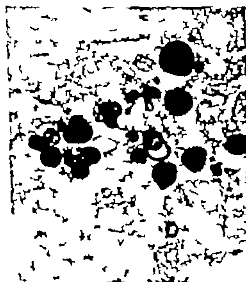


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cordance with those found in previous studies. (Bergman and an der Linden 1965 1966 a) The thyroxine treated hamsters showed marked f tty degeneration of the liver These changes were less pronounced in animals subseq ently fed normal chow and in those fed the unsupplemented diet 295 whereas they had disappeared completely in animals fed this latter diet supplemented w th cholestyramine

Electron microscopy After 2 weeks treatment w th the diet containing thyroxine,

the parenchymal cells of the liver started to show numerous lipid droplets. Moreover clusters of dense osmiophilic bodies were seen which often showed characteristic vacuolated appearance and myelin figures consisting of dense concentric lamellae (Fig. 4 and 5). In sections incubated in Gomori medium they showed positive acid phosphatase reaction products. These morphological changes were found almost exclusively in the thyroxine treated animals and increased in frequency with the duration of the treatment. Numerous clusters of these osmiophilic bodies could still be seen in all the animals which after treatment with thyroxine were fed with other diets during one month. The lipid droplets, on the other hand, diminished in number or even disappeared when the diet was changed.

Discussion

In a previous stud[y] it was found that the addition of 1 % cholestyramine to the diet resulted in a decreased bile acid concentration in the bile of guinea pigs (Schoenfield and Sjövall 1966 a). The cholesterol concentration increased slightly and under certain dietary conditions gallstones containing cholesterol were formed. The present study has shown that cholestyramine when added to a cholesterol gallstone inducing diet does not lower the bile acid concentration in the bile of hamsters. On the contrary, there was an increase in the bile acid/cholesterol ratio in the bile and cholesterol stones were not formed (Bergman and van der Linden 1966 b, 1967). It is possible that this difference reflects a different response in the two species to the increased loss of bile acids via the feces (Tennent et al. 1960). Thus, in response to biliary fistula some species e.g. the rabbit (Lindstedt and Sjövall 1957) increase their bile acid production more slowly than others e.g. the rat (Eriksson 1957). The results of exp. 2 indicate that the hamster can increase the bile acid concentration in the hepatic bile after the initial loss of the bile acid pool through a fistula. In long term experiments with cholestyramine, however, a new equilibrium between bile acid turnover time and pool size will be established and the rate of turnover may not necessarily influence the biliary bile acid concentration.

The concentration of bile acids in hepatic bile depends largely on the enterohepatic recirculation of the bile acid pool (for further details the reader is referred to the excellent review by Danielsson 1963). If one assumes that cholestyramine affects the enterohepatic circulation of different bile acids differently, its effect on the biliary bile acid concentration in bile could depend on the nature of the bile acids in the species studied. In guinea pigs chenodeoxycholic and 7-ketodeoxycholic acids (conjugated with glycine or taurine) are the predominant bile acids, and cholestyramine lowers the concentration of both these acids (Schoenfield and Sjövall 1966 a). In hamsters fed diet 295, cholic and chenodeoxycholic acids (conjugated with glycine or taurine; Prange et al. 1962) are the predominant bile acids. In these animals cholestyramine decreases the concentration of chenodeoxycholic acid markedly while the cholic acid concentration is correspondingly increased (cf. Fig. 2 and 3). The result is an unchanged concentration of total bile acids. The change in the proportions of cholic and chenodeoxycholic acid during

the elimination of the bile acid pool through a biliary fistula (observed in animals fed diets 295 and HK) also indicates that there may be a difference in the enterohepatic circulation of these acids.

Whatever the reason may be for the different response of hamsters and guinea pigs to cholestyramine it is clear that the ion exchanger alters the bile acid pattern in hamster bile. A similar drastic change has not been observed by Dam and coworkers in studies of bile acids in hamsters fed gallstone inducing and dissolving diets (Prange *et al.* 1962). It is therefore less likely that the increased proportion of cholic acid on the cholestyramine supplemented diet is directly related to the dissolution of cholesterol stones. In pure solutions chenodeoxycholic acid is a more potent solubilizing agent than cholic acid but this cannot be compared with the conditions in bile where cholesterol is held in solution in a mixed micelle containing bile salts and lecithin (Hofmann and Small 1967). In agreement with the extensive data from Dam's group the ratio total bile acids/cholesterol was lower in animals fed the gallstone inducing diet than in those given the "curative" diet (Prange *et al.* 1962).

In addition to cholic, chenodeoxycholic and deoxycholic acids previously determined by Prange *et al.* (1962) a number of mono- di- and trisubstituted bile acids were identified. Of these, 7 ketodeoxycholic and 7α,12α-dihydroxy 3-keto-5β-cholanoic acids were the predominant compounds. Small amounts of a 5α-cholanoic acid, allocholic acid were found in some samples. There was no consistent difference in the concentration of these compounds between the various groups of animals.

Like cholestyramine, L thyroxine gave an increased ratio of cholic to chenodeoxycholic acid. Perhaps the effects of the drug and the hormone are analogous in that both stimulate the bile acid production (Tennent *et al.* 1960 Huff *et al.* 1963 Strand 1963). In rats, cholestyramine increases the fecal excretion particularly of dihydroxy bile acids (Huff *et al.* 1963) triiodothyronine increases the production and pool of chenodeoxycholic acid —3 times, and the biliary concentration of this acid is greatly increased on thyroid administration (Eriksson 1957 Strand 1963). This is the reverse of what is found in hamsters in the sense that cholestyramine and thyroxine decrease the biliary chenodeoxycholic acid concentration and indicates a species difference in the response to thyroxine.

In exp. 3 *the morpho-histochemical degenerative changes found in the hepatic* parenchymal cells seemed to be exclusively bound to the thyroxine treated group. The significant increase in the number of dense osmiophilic bodies, found at electron microscopy in the hepatic parenchymal cells is believed to represent advanced focal cytoplasmic degradation. This phenomenon is a common observation reflecting a non specific type of hepatocellular reaction to injury (Hruban *et al.* 1963 Confer and Strenger 1964 Biava 1965). Comparable ultrastructural changes have been observed in the liver cells of thyroid treated rats (Schultz *et al.* 1957. It may also be of interest to point out that thyroxine potentiates the effect of certain hepatotoxic agents (Black-Schaffer Johnson and Gobbel 1950 Calvert and Brod 1961).

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The terminology of the observed vacuolated osmiophilic dense bodies is confusing. DeDuve (1963) who originally developed the lysosome concept proposed the descriptive term autophagic vacuole in order to avoid problems concerning their relationship to lysosomes. The intense osmiophilia of these bodies and their tendency to form myelin figures indicate that they consist predominantly of lipids and that they may represent an example of lipophanerosis at the subcellular level.

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References

BERGMAN F and W AN DER LINDE. Influence of D-thyroxine on Gallstone Formation in Hamsters. A t chir scand 1965. 129 547—552.
BERGMAN F and W VAN DER LINDEN Further Studies on the Influence of Thyroxine on Gallstone Formation in Hamsters. Acta chir scand 1966 a. 131 319—328.
BERGMAN F and W VAN DER LINDEN Influence of Cholestyramine, a Bile Acid Sequestrant, on Gallstone Formation in Hamsters. Acta chir scand. 1966 b. 132 724—730.
BERGMAN F and W A DER LINDE , Diet-induced cholesterol gallstones in hamsters prevention and dissolution by holestyramine. Gastroe terology 1967 53 418—421
BU VA C Electron microscopic studies on periodic acid-Schiff-positiv nonglycogenic structures in human liver cells. Amer J Path. 1965. 46 435—466.
BLACK SCHAF ER B D S. JOHNSON and W G GOBBEL J Experimental total midzonal hepatic necrosis. Amer J Path. 1950 26 397—409
CAL ER D N and T M. BRODY The effect of thyroid function upon carbon tetrachloride hepatotoxicity J Pharmacol. p. Ther 1961 134 304—310.
CON ER. D B and R. J STENGER. The evolution of lysosomes in hypoxic liver parenchyma as seen with the electron microscope. Amer J Path. 1964 45 533—546.
DAM H Experimentelle alimentäre Erzeugung on Gallensteinen. M d. u. Ernäh 1963 6 206—210 23 — 41 and 263—266.
DANIELSSON H P se t stat of sear h t bolism and cretio f hol sterol In Advances Lipid Research (eds. R. Paoletti and D Kritchevsky) Academic Press, New York. Vol 1 p 335—385 1963
DEDU E C The Lysosome Conc pt 1 Lysosomes. Ciba Foundation Symposium De Reuck, A. V S and Cameron, M. P eds.) Little Brow & Co., Boston. 1963 1- 35
E EROTH P B GORDON R BYHAGE and J SJÖ ALL, Identification of mono- and dihydroxy bile acids in human feces by gas-liquid chromatography and mass spectrometry J Lipid Re 1966 311—323
ERICSSON J L E and B F TRUMP Electron Microscopic Studies of the Epithelium of the Proximal Tubule of the Rat Kidney I The I tracellular Localization of Acid Phosphatase Lab I st 1964 13 1427—1456
ERIKSSON S Influenc of thyroid activity on excretion of bile acids and holesterol in the rat. Proc Soc p Biol. N Y 1957 94 582—584
HOFMA A. F and D M SMALL. Detergent properties of bile salts correlation th physiological function. A Re M d 196 18 333—3 6.
HA AS Z B SPARGO H. SWIFT R W WISSLER and R. G KLEINFELD Focal toplasmic degradation Amer J Path 1963 42 (5 —684
HUFF J W J L GILFILLA and V M H NT Effect of holestyramine bile acid binding polymer on plasma holesterol and fecal bile acid excretion in the rat. Proc Soc p Biol (N Y) 1963 114 352—355
LINDSTEDT S and J SJÖVALL. On the formation of deoxycholic acid from cholic acid in the rabbit. Act hem. scand 1957 11 421—426.
LUFT H. J Improvements in epoxy resin embedding methods. J biophy biochem Cytol 1961 9 409—414
MAKITA M. and W W WELLS, Quantitativ analysis of fecal bile acids by gas-liquid chromatography Analyt Biochem. 1963 5 523— 530
MILLER, F Acid phosphatas localizatio renal p otei bsorptio d plet I Electron Microscopy Fifth International Congress on Electron Microscopy Philadelphia Pennsylvania, August 29 to September 5 1962 edited by Breese S S J Vol. 2, p Q-2 New York, Academic Press, Inc 1962.

Prange, I., F. Christensen and H. Dam. Alimentary production of gallstones in hamsters. 11. Relation between diet and composition of the bladder bile. *Z. Ernährungsw.* 1962, *3*, 59—78.

Schoenfield, L. and L. Sjövall, Bile acids and cholesterol in guinea pigs with induced gallstones. *Amer. J. Physiol.* 1966 a, *211*, 1069—1074.

Schoenfield, L. and J. Sjövall, Bile acid composition of gallstones from man. *J. Lab. clin. Med.* 1966 b, *68*, 186—194.

Schultz, H., H. Löw, L. Ernster and F. S. Sjöstrand, *Elektronenmikroskopische Studien an Leberschnitten von Thyroxin-behandelten Ratten.* In: Electron Microscopy. Proc. Stockholm Conf. Academic Press Inc., New York. 1957, 134—137.

Strand, O. Effects of D- and L-triiodothyronine and of propylthiouracil on the production of bile acids in the rat. *J. Lipid Res.* 1963, *4*, 305—311.

Tennent, D. M., H. Siegel, M. E. Zanetti, G. W. Kuron, W. H. Ott and F. J. Wolf. Plasma cholesterol lowering action of bile acid binding polymers in experimental animals. *J. Lipid Res.* 1960, *1*, 469—473.

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The Influence of Hypoxia on the Spontaneous Thalamic and Cortical Barbiturate Spindles

By

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Abstract

EIDHOLM, B. K. JUNGE and O. SVEEN *The influence of hypoxia on the spontaneous thalamic and cortical barbiturate spindles* Acta physiol. scand. 1968 74 492—500

Spontaneous barbiturate spindles in the thalamus and the cortex were recorded in cats anesthetized with barbiturates and subjected to hypoxia. During stagnant hypoxia produced by pharmacological reduction of the blood pressure there was no spindle activity when the blood pressure was below 60 mm Hg. In hypoxic hypoxia, the spindles disappeared after the oxygen content of the nitrogen-oxygen mixture had been reduced to 4.5 per cent. In this condition the blood pressure was always at or above 80 mm Hg and very often above 100 mm Hg. The lack of spindles was probably the result of the combined effect of changes in the blood pressure and the local tissue oxygen level.—Before and after the state of no spindle activity there was a period of high synchrony characterized by low degree of activity between the spindles and high-voltage waves within the spindles. Except for the abrupt disappearance and reappearance of the spindles, the degree of synchrony was the only parameter of barbiturate spindle activity that varied during hypoxia.—The results suggest that the mechanism of spindle activity may operate with a relatively small number of cells. A more complex neuronal system with a greater number of synapses may be supposed to be more susceptible to severe degrees of hypoxia.

Numerous studies have shown that acute hypoxia or anoxia lead, through intermediate stages, to isoelectric flat EEG (Meyer and Gastaut 1961) but it seems that no one has yet studied the relation between thalamic spindles recorded by microelectrodes and acute hypoxia. Since the thalamic rhythmic activity controls the corresponding cortical waves (Bremer 1937, Andersen, Andersson and Lømo 1967a, b) and since the thalamus is the only subcortical source of such rhythm (Junge and Sveen 1968) this relation is of special interest.

It has been suggested that individual nerve cells have an intrinsic rhythmic property (Bremer 1949, 1953, Clare and Bishop 1956). Another explanation of the rhythmic activity of the brain is that the cycle of the rhythm is set by the time of propagation along closed self-re-exciting chains of neurones (Lorente de Nó 1938, Chang 1950, Burns 1951, 1958). For example Chang claimed that the rhythmic

thalamic discharge was due to a reverberatory thalamo-cortical circuit because it was abolished by removal of the cortex or by interruption of the cortico-thalamic connections. However this statement is contradicted by many reports (Adrian 1941 1951 Morison *et al.* 1943, Bremer and Bonnet 1950 Bremer 1955 Galambos *et al.* 1952, Andersen and Sears 1964 Andersen *et al.* 1964)

A probabl explanation of the thalamic spindles is the inhibitory phasing theory (Andersen and Eccles 1962, Andersen and Sears 1964) According to this theory interneurones, synaptically excited by axon collaterals of thalamic neurones, induce inhibitory postsynaptic potentials in those neurones. Each interneurone has inhibitory synapses on many neurones which are distributed over an extensive area, which is a necessary condition for synchronous activity Andersen and Sears (1964) have shown that the positive waves in extracellular recording of thalamic spindles are due to nearly simultaneous inhibitory postsynaptic potentials in many thalamic neurones. The neurones discharge on the terminal phase of their inhibitory post synaptic potentials with a group of spikes. Through the recurrent pathways, a new positive wave is generated and the cycle repeats itself.

The narrow loop postulated by the inhibitory phasing theory should be less susceptible to hypoxia than the more complex loops of self re-exciting chains that ha e been suggested to account for such rhythmic acti ty (Burns 1951 Chang 1950) Specifically it would be of interest to see whether the frequency of the thalamic rhythmic acti ty slows down during increasing hypoxia as it does under deepening barbiturate anesthesia.

Methods

Young and adult cats weighing 2.5—3 kg were used. They were anesthetized by intraperitoneal injections of pentobarbital sodium, 30 mg/kg Light anesthesia was maintained by i. injection of small doses of barbiturate when necessary The animal's head was placed in head-holder. The skull was removed to expose the convexity of the cerebral cortex After removal of the dura, the middle portions of the lateral, suprasylvian and ectosylvian gyri were removed by suction The rostral border of the cortical ablation was restricted so that all thalamo-cortical fibres to the somatosensory area I and II were lef intact. After exposure of the lateral entricle, the fimbria and the hippocampus were removed with glass sucker thus exposing the dorsal surface of the thalamus in the telo-diencephalic fissure I some cats both thalami ere exposed.

Recording from the thalamus was made with glass microelectrodes with 4M NaCl (1 –4.5 MΩ) which were placed in micromanipulator for ertical penetration of the thalamus The signals obtained from the micro-electrodes were fed through cathode follower and low-level c. amplifier befor being recorded and displayed on an oscilloscope and an ink writer Surface potentials from the somato-sensory cortex were recorded with platinum wire electrodes ending in small ball. They were amplified and displayed in the same manner as the micro-electrode signals

The blood pressure of the femoral artery was recorded by pressure transducer and displayed on an ink writer End-tidal pCO was continuously measured by an nfrared carbon dioxide analyser Samples were taken from the trachea by narrow tube through the tracheal cannula. The output of the analyser was recorded on the ink writer The relati oxygen tension of thalamic tissue was measured by open polarography A platinum electrode was placed either in the contralateral thalamus or in the same halamus as the micro-electrodes. A potential of +0 6 olts relati to earth was applied to this electrode The oxygen tension in the tissue is proportional to the current flowing from the electrode into the tissue. The output of the polarographic device was d.c recorded and displayed on an oscilloscope screen and photographed.

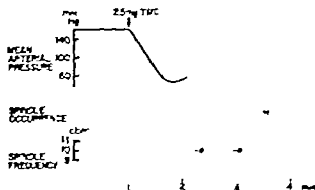


Fig. 1 Effect of trimethaphan camphosulfonat (TMCS) on mean arterial pressure spindle occurrence and spindle frequency Cat, barbiturate anesthesia. In this and the following figures, the blood pressure line is drawn from the original curve through the point representing the mean arterial pressure (M.A.P.)

The animal was curarized by intravenous injections of Flaxedil and artificially ventilated by a pump. A mixture of oxygen and nitrogen was supplied to the animal through a mixer at a constant pressure.

In the experiments with pharmacological reduction of the blood pressure neither gas measurements nor artificial respiration were undertaken. The reduction was brought about by injection of the ganglion-blocking agent trimethaphan camphosulfonat (Arfonad®). When necessary the blood pressure was increased by injecting the vasopressor substance metaraminol (Aramine®).

Results

Stagnant hypoxia. The reduced blood flow through the brain that is produced by [trimeth]aphan camphosulfonate (TMCS) leads to stagnant hypoxia. An injection of 25 mg TMCS was followed by a relatively rapid change in the mean arterial pressure (M.A.P.) from about 160 to about 40 mm Hg (Fig. 1). When the decrease in M.A.P. was rapid the spindles tended to disappear at about 100 mm Hg. In the experiment illustrated in Fig. 1 they reappeared after about 3/4 min when the M.A.P. reached 60 mm Hg after the initial dip. There was no systematic change in the spindle frequency during the experimental run. In the present work, spindle frequency is taken as the frequency of the waves within a spindle. The frequency was counted only for spindles where the waves could be clearly distinguished from the background noise.

Fig. 2 shows the results of an experimental run on another animal. An injection of 5 mg TMCS caused a rapid change in the M.A.P. from about 140 to about 60 mm Hg. After 2 1/2 min. the blood pressure was transiently raised by an injection of 0.125 mg metaraminol. This dosage was repeated and the M.A.P. each time increased to about [illegible]0 mm Hg. Except for the initial disappearance at about 100 mm Hg, the spindle activity was absent when the M.A.P. was at or below 60 mm Hg. There was no systematic change in the spindle frequency throughout the run.

The data in Fig. 3 are taken from the same kind of experiments as those shown in Fig. 1 and 2. The spindle interval and the spindle duration are plotted against the level of the M.A.P. There was no significant relationship between these parameters.

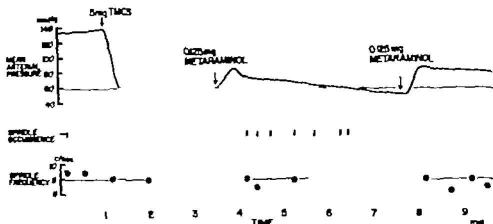


Fig. 2. Effect of trimethaphan camphosulfonate (TMCS) on mean arterial pressure spindle occurrence and spindle frequency. The blood pressure was raised by injections of metaraminol.

Hypoxic hypoxia. This state was produced by reducing the oxygen content of the nitrogen-oxygen mixture. A reduction of the available oxygen from 30 to 7 % was followed by short rise and a subsequent fall in the M.A.P. (Fig. 4). During the first 8 min of this fall, there was no obvious change in the spindle occurrence or spindle frequency. Available oxygen was then further reduced from 7 to 4.5 %. When the M.A.P. reached about 75 mm Hg the thalamic spindles disappeared and were replaced by random neuronal activity. Subsequently the animal was pressure ventilated with 50 % available oxygen for 1.6 min and further ventilated at the usual pressure with 30 % oxygen. This brought about an increase in the M.A.P. to about 110 mm Hg for 4 min, but no spindles occurred. Consequently the dis-



Fig. 3. Mean arterial blood pressure is plotted against spindle interval and spindle duration, respectively. The data are taken from the same kind of experiments as those shown in Fig. 1 and 2.

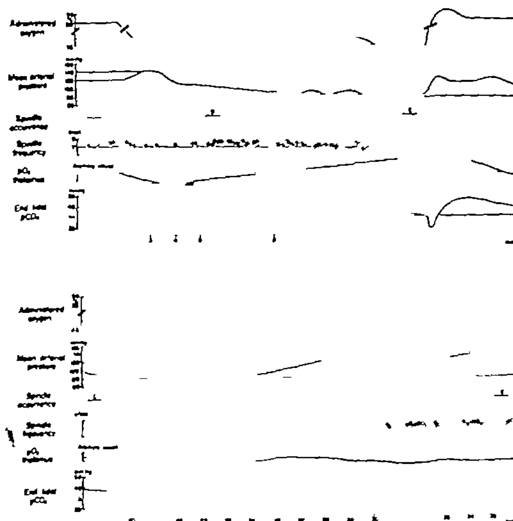


Fig. 4. Hypoxic hypoxia. Effect of changes in available oxygen on mean arterial pressure, spindle occurrence and spindle frequency. Records of pO_2 in the thalamus and pCO_2 of the end-tidal expiration air are also shown.

appearance of spindle activity is not exclusively controlled by the M.A.P. but may in addition depend upon the local tissue oxygen level.

After 30 % oxygen at the usual pressure had been maintained for almost a quarter of an hour the spindles re-appeared. At that time the M.A.P. had increased to and remained stable at 125 mm Hg for a period of 6 min. There were no systematic changes in the spindle occurrence or spindle frequency either in the initial hypoxic period or during the recovery. The spindle activity showed an abrupt disappearance and reappeared equally suddenly.

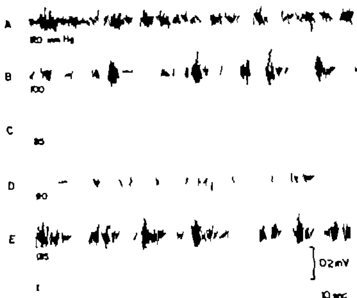


Fig. 5. Excerpts of the thalamic recording t the times indicated by bars in Fig. 4. Only the slow wa components are seen.

Reduction of the available oxygen from 30 to 7 % caused a fall in the relative oxygen tension in the thalamus. The subsequent rise in the curve is an artefact that may be ascribed to edema caused by the hypoxic condition. Beyond 19 min, the relative oxygen tension of the thalamus was quite constant, but cannot be compared to the prehypoxic level due to the change in the eference level caused by the edema.

In order to ascertain that changes in pCO did not influence the results, the carbon dioxide tension in the end tidal expiration air was monitored. The pCO_2 was found to vary significantly only during and shortly after th increased pressure ventilation.

Fig. 5 shows excerpts of the thalamic recording at the times ndicated by bars in Fig. 4. Only the slow wave components are seen. Between the prehypoxic state (A) and the isoelectric state (C) there was a period of high synchrony (B) characterized by low degree of activity between the spindles and high- oltage wa es within the spindles. These features were also apparent in the period following the reappearance of the spindles (E). The stage of high synchrony (B and E) is similar to that found during optimal barbiturate anesthesia. Lowering the oxygen tension of the tissue probably reduces the number of neurones participating in the afferent barrage. Since this has the same effect as a partial deafferentation, a higher degree of synchrony will result, paralleling the find ng of Bremer (1953) after deafferentation by transection of the brain stem.



Fig. 6. Effect of changes in available oxygen on mean arterial pressure, spindle occurrence, spindle frequency and primary evoked potentials. Record of pCO_2 of the end-tidal expiration air is also shown.

All figures presented here show only thalamic spindles but parallel findings were obtained by the simultaneous recording of cortical spindles.

The experimental run illustrated in Fig. 6 is from a different cat than that reported in Fig. 4 and 5. After the oxygen content of the inspired air was reduced directly from 30 to 4.5 % the spindles disappeared for about 1 min. This seems to be the same phenomenon as the lowered spindle activity around 4 min in Fig. 4 and may be due to a lag in the autoregulation of the cerebral circulation. Evoked potentials were recorded from the somato-sensory cortex in response to electrical shocks to the contralateral forelimb (Fig. 6). The evoked cortical potentials reappeared later than the thalamic spindles, but it was hard to decide definitely whether they also returned later than the cortical spindles. Because of this uncertainty we will not draw any conclusions on this point.

Discussion

According to the inhibitory phasing theory the spindle mechanism requires the nearly simultaneous discharge of many neighbouring neurones and the operation of a widespread recurrent inhibition. Both projection neurones and interneurones are supposed to partake. It was, therefore somewhat surprising that the mechanism of the spindl activity was so resistant to severe degrees of hypoxia. However a more complex neuronal chain with a greater number of synapses in the circuit would be less resistant. These results open th possibility that thalamic rhythmic activity may operate with a relatively small number of cells. The all-or-nothing effect found with regard to the presence of spindles probably reflects the autoregulation of the brain circulation. Except for the abrupt disappearance and reappearance of the spindles, degree of synchrony was the only parameter of barbiturate spindle activity that varied during hypoxia, but this parameter can hardly be of any practical use as an indicator of hypoxia.

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References

Adrian, E. D. Afferent discharges to the cerebral cortex from peripheral sense organs. *J. Physiol.* (Lond.) 1941 *100* 159—191.
Adrian, E. D. Rhythmic discharges from the thalamus. *J. Physiol.* (Lond.) 1951 *115* 9—10 P.
Andersen, P. S. A. Andersson and T. Lømo, Nature of thalamo-cortical relations during spontaneous barbiturate spindle activity. *J. Physiol.* (Lond.) 1967 *192* 283—307.
Andersen, P. S. A. Andersson and T. Lømo. Some factors involved in the thalamic control of spontaneous barbiturate spindles. *J. Physiol.* (Lond.) 1967b. *192* 257—281.
Andersen, P. C. McC. Brooks, J. C. Eccles and T. A. Sears. The ventro-basal nucleus of the thalamus: potential fields, synaptic transmission and excitability of both pre-synaptic and post-synaptic components. *J. Physiol.* (Lond.) 1964. *174* 348—369.
Andersen, P. and J. Eccles, Inhibitory phasing of neuronal discharge. *Nature* (Lond.) 1962. *196* 645—647.

ANDERSEN, P. and T. A. SEARS, The role of inhibition in the phasing of spontaneous thalamo-cortical discharges. *J. Physiol.* (Lond.) 1964, *173* 459—480.

BREMER, F. L'activité cérébral au cours du sommeil et de la narcose. Contribution à l'étude du mécanisme du sommeil. *Bull. Acad. Roy. Méd. Belg.* 1937 *2* 68—86

BREMER, F. Considérations sur l'origine et la nature des ondes cérébrales. *Electroenceph. clin. Neurophysiol.* 1949 *1* 177—193

BREMER, F. Physiologie du corps calleux. *J. Physiol.* (Paris) 1953 *45* 52—53.

BREMER, F. and V. BONNET Interprétation des réactions rhythmiques prolongées des aires sensorielles de l'écorce cérébrale. *Electroenc ph. cl Neur physiol* 1950 *2* 389—400.

BURNS, B. D., Some properties of isolated cerebral cortex in the unanaesthetized cat. *J. Physiol.* (Lond.) 1951 *112* 156—175

BURNS, B. D. *The mammalia cer al cort x.* London. Ed ard Arnold. 1958.

CHANG, H. T. The repetitive discharges of corticothalamic reverberating circuits. *J. Neurophysiol.* 1950. *13* 235—258.

CLARE, M. H. and G. H. BISHOP Potential wav mechanisms in cat cortex. *Electroenc ph cl Neu physiol.* 1956 *8* 583—602.

GALAMBOS, R., J. E. ROSE, R. B. BROMILEY and J. R. HUGHES, Microelectrode studies on medial geniculate body of cat. II Response to clicks. *J. Neurophysiol.* 1952. *15* 359—380.

JUTOR, K. and O. S EN Exclusiv thalamic location of subcortical spontaneous barbiturate spindles. *Act physiol. scand.* 1968. *73* 22—31

LORENTE DE NÓ R., Analysis of the activity of the chains of internuncial neurons. *J. Neurophysiol.* 1938. *1* 207—244

MEYER, J. S. and H. GASTAUT *Cerebral oxia nd the ele troenc phalogram.* Springfield. Charles C. Thomas. 1961

MORISON R. S. K. H. FINLEY and G. N. LOTHROP Spontaneous lectrical activity of the thalamus and other forebrain structures. *J. Neurophysiol.* 1943 *6* 243—254

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Inhibitory Action of Adrenergic Blocking Agents on Reuptake and Net Uptake of Noradrenaline in Nerve Granules

By

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Abstract

EULER, U. S. v and F LISHAJKO. *Inhibitory action of adrenergic blocking agents on reuptake and net uptake of noradrenaline in nerve granules.* Acta physiol. 1968. 74 501—506.

The effect of various adrenergic blocking agents on the reuptake and net uptake of noradrenaline (NA) in isolated adrenergic nerve granules has been studied. All of the agents tested exerted an inhibitory action on the spontaneous release rate of NA and also on the reuptake of ^{3}H labelled NA from a medium containing ... The highest activity was shown by the -blockers dihydroergotamine and ... blockers were almost as active. No significant difference in inhibitory ... between the l- and d-isomers of propranolol and Aptin. In the presence of ... M—10 M the net uptake of NA in partially depleted granules was ... same blocking agents as were most active on the reuptake. The ... previous finding that adrenergic blocking agents, both of the α- and β-type ... in nerve granules. The possible mediation of transmitter release and uptake ... tors is discussed.

It has been observed that, among other biologically active ... adrenergic blocking agents inhibit uptake of labelled noradrenaline ... organs in vivo (Hertting, Axelrod and Whitby 1961 Lindmar ... Lemsen 1963 Gillespie and Kirpekar 1965) This effect ... to an action on the axon membrane However studies on ... have shown that for instance phenoxybenzamine also acts ... of NA in this system (Euler and Lishajko 1961) In a ... vestigated more systematically the effect of adrenergic bl... α- and β-blocking type, on the spontaneous release and ... take of NA in partially NA-depleted adrenergic nerve g... 1966) It was then observed that all of the adrenergic bl... the release and the uptake of NA although to varying ... us too regular a phenomenon to be only a coincidence

The results were taken to indicate that the intera... in granules as well as in effector cells are related ...

by the same group of substances. If this were so, the nerve granules might offer a suitable material for studying by analogy uptake and binding mechanisms which would be valid in principle also for receptors of the effector cells, which ordinarily do not lend themselves readily to such studies.

As shown previously the rate of spontaneous release of NA from adrenergic nerve granules at 20° or 37 depends, among other factors, on the concentration of NA in the medium, owing to reuptake (Euler Stjärne and Lishajko 1963 Euler and Lishajko 1967a) The reuptake can be estimated by measuring the incorporation of labelled NA into the granules, even though a certain reuptake of nonlabelled material apparently occurs (*cf* Euler and Lishajko 1967a) A suitable concentration range of NA in the medium for studying reuptake is 1—3 × 10 ⁶ M. When partially depleted granules are incubated in the presence of ATP Mg^{2+} and NA, a large net uptake of NA takes place (Euler and Lishajko 1963 Stjärne 1964)

The present report deals with the effect of a series of α- and β-blockers on the reuptake of NA in adrenergic nerve granules, studied by measuring the incorporation of radioactive NA, and on the net uptake of NA in partially depleted granules.

Methods

Nerv granules were obtained by homogenization of bovine splenic nerves in ice-cold phosphate buffer and removal of coarse particles by centrif gation for 10 min t 9000 × *g* The granule suspension in the supernatant served as material for incubation in isotonic potassium phosphate t pH 7.5 for 60 min at 20 C. The concentration of free NA in the medium under these conditions was 1—3 × 10 ⁶ M. After incubation the suspension was centrifuged for 30 min at ,000 × *g* and NA determined fluorimetrically in the sediment.

NA in the supernatan was adsorbed on alumina, eluted with 0.25 NA acetic acid and etermined fluorimetrically

Tw kinds of experiments were made. I the first series the granule suspension was incubated for 60 min t 20 in the presence of blocking gent in the concentrations 3 × 10 M 10 M and 3 × 10 M and dl- H NA (total NA concentration 1—3 × 10 M) The incorporation of tritiated NA (by reuptake) was det rmined and expressed as percentage of the control alue

I the second series, granules were partially depleted by incubation for 10 min at 37 and the net ptake of NA determined after further ncubation for 30 min t 20 in the presence of the blocking agent and NA 5 × 10 —10 M, ATP 5mM, and $MgCl_2$ 3 mM The relativ net uptake was expressed as per cent of the ATP-dependent ptake *i.e* the ptake excess of that observed w th addition of NA alone. This way of calculating the ptake differs slightly from that used in the previou st dy Euler and Lishajko 1966)

Radioactivity was determined in aliquots of the same extracts as used for NA estimation in a *Packard liquid scintillation spectrometer*

The incorporation f dl-³H NA added to the ncubation medium in concentration of about 5 × 10 M was estimated as the ratio of the specific activities of NA in the sediment and in the supernatant after high speed centrifugation (SAsed/SA r Stjärne 1964

Most of the adrenergic blocking agents were added in concentration of 3 × 10 M. I most experiments the granules were preincubated with the blockers for 30 min at 2 C The gift of the following drugs is gratef lly acknowledged Butoxamine and BW 61-43 (Burroughs Wellcome Ltd., Tuckahoe N Y) Orciprenaline (Alupent) and KO-592 (Boehringer Sohn Plegicil (Pharmacia, Uppsala) l- and d-Propranolol and Pronethalol (Alderin) (I C I Scandia, Göteborg) l- and d-H 56/28 (Aptin) 1 (o-allyl phenoxy) 3-isopropylamino- -propanol (AB Hässle Göteborg) MJ 1998 and 1999 (Mead Johnson Research Center E ansville) Azapetine (Ilida) (Hoffmann-La Roche Basel) SY 28 (N-α-naphthylmethyl-N-ethyl-β-bromoethylamine) (Parke-Davis & Co Ann Arbor) Hydergi (Dihydroergotamine DHE) (Sandoz, Basel) Dichloroisoproterenol (Lilly Research Laboratories, Indianapolis Phentolamine (Regitine) (CIBA, Basel Phenoxybenzamine (Dibenyline) (Smith, Kline & French, Philadelphia) Chlorpromazine (Hibernal) (I C I) 1 pes (Sch & C Milano

TABLE I. Left panel: Incorporation of dl-H-NA in adrenergic nerve granules incubated in phosphate buffer pH 7.5 for 60 min at 20 in the presence of adrenergic blocking agents, in per cent of control. NA concentration in medium $1-3 \times 10^{-8}$ M. Right panel: Uptake of NA in partially NA-depleted adrenergic nerve granules incubated 30 min at 20 in th presence of 5×10^{-5}—10^{-} M NA and ATP+Mg^{+} 3 mM and blocking agents, in per cent of control

	Incorporation of dl- H NA in granules in per cent of control			Net uptake of NA in granules in per cent of control		
	3×10^{-4}M	10^{-} M	3×10 M	3×10^{-4}M	10^{-4}M	$3 \times 10^{-}$ M
Hydergin	26	—	—	25	—	—
Dihydroergotamine	27	5	<5	26	—	—
Regitol	21	9	<5	32	<10	<10
Azapetine	31	—	<5	45	—	<10
Chlorpromazine	24	7	<5	52	16	—
Pronethalol	22	—	<5	—	—	<10
Dichloroisoproterenol	29	—	<5	—	—	<10
l-propranolol	32	—	<5	55	15	<10
d-propranolol	29	—	<5	—	—	<10
l-Aptin	43	27	<5	78	32	12
d-Aptin	37	24	<5	82	36	<10
KÖ-592	57	25	5—10	—	23	16
Phenoxybenzamine	28	—	5—10	—	—	23
Phentolamine	94	—	5—10	—	—	26
SY 28	63	—	13	—	—	47
Butoxamine	—	—	32	—	—	50
MJ 1999	87	—	63	—	—	58
BW 61—43	—	—	28	—	—	64
Inpea	83	70	37	—	96	76
MJ 1998	—	—	—	—	—	91
Orciprenaline	—	47	31	—	—	94

Results

1 *Eff ct f ad energic blockers on reuptak of NA*

The incorporation of tritiated NA, by reuptake in th granules, in per cent of that of the control, is shown in Table I During the prevailing experimental conditions the incorporation of labelled NA amounts to about one third of the total NA in the granules. As seen in Table I, a large number of blocking agents, both of the α- and β-blocking kind, inhibit reuptake to more than 90 per cent concentration of 3×10 M and to 40—80 per cent at 3×10 M

It is further seen that in contrast to the antiadrenergic effect on the effector cell receptors, there is practically no difference in ction between the l and d-forms of propranolol and of Aptin (Åblad Brogård and Ek 1967)

As noted in the previous communication (Euler and Lishajko 1966) the β-blockers Inpea and MJ 1999 have a relatively small effect on this system.

We have also tested a dibenamine congener GD-131 (N-ethyl N-cyclohexyl-β-chloroethylamine) (kindly placed at our disposal by Dr M. Nickerson, Montreal) on the release and reuptake of NA in granules. This compound has a rather weak α-blocking action even in a concentration of 4×10^{-4} M on isolated organs. When tested on the NA reuptake in isolated granules in concentrations up to 3×10^{-4} M GD-131 inhibited the uptake less than 10 per cent. This suggests that the inhibitory effect of other β-haloalkylamines on the reuptake and net uptake of NA in granules is not unspecific in nature but may be related to their α-blocking effect.

II *Effect of adrenergic blockers on net uptake of NA*

In these experiments NA was added both in a relatively high concentration, 10^{-4} M which produces some net uptake even without addition of ATP and in a lower concentration, 5×10^{-6} M As seen in Table I the net uptake of NA is strongly inhibited by a number of both α- and β-blockers. The inhibitory action of the blockers was somewhat reduced at the higher NA concentration, probably indicating a competitive action.

Discussion

Inhibition of uptake of NA in nerve granules has been observed for a number of compounds (*cf* Euler and Lishajko 1965) such as reserpine, prenylamine (Euler Lishajko 1968a) desmethylimipramine and some indirectly acting amines er and Lishajko 1968 b) in addition to a variety of uncouplers and metabolic itors (Euler and Lishajko to be published) Since these compounds do not generally exert antiadrenergic actions on effector cells their mode of action is presumably of another kind than that exerted by the adrenergic blocking agents.

Inhibitors of reuptake and net uptake of NA may either enhance or inhibit the release rate of NA from the granules or leave it unaffected. To the first mentioned group belong some indirectly acting amines (Euler and Lishajko 1968 b) but in most other instances the inhibitors of NA reuptake and net uptake also retard the spontaneous release of NA from the granules, thus reducing their ability for uptake of NA.

Since the net uptake of NA in partially depleted nerve granules is strongly enhanced in the presence of ATP and Mg^{2+} (Euler and Lishajko 1963) we ha used this system for studying the effect of adrenergic blocking agents on the NA ptake From the results it is evident that both α- and β-blocking agents can exert a potent inhibitory action on the net uptake of NA in granules.

It is not possible from the degree of inhibition observed with the different blockers to conclude whether th receptor mechanism of the granules is mainly of the α- or β-type The possibility remains that the granules possess a receptor mechanism which is less differentiated than that of the effector cells and therefore can be affected by adrenergic blocking agents of both kinds.

As observed in the present study there is no definite difference in the action on reuptake of NA of the l- and d forms of propranolol and Aptin. This might also be an indication that the binding of the transmitter in the granule is less specific, or differentiated, than in the effector cells. As pointed out by Moran (1966) the interaction between agonist and antagonist on receptor systems may be considerably more complicated than usually assumed. On the other hand there is a general tendency for those blockers which have a weaker effect on effector cells also to be less active on the NA uptake in granules. This is well borne out by the finding that a dibenamine-congener GD-131 with very little α-blocking action, is also almost inactive on the granules.

Also in human blood platelets the uptake of dl-^{3}H NA is strongly inhibited by adrenergic blocking agents of the α- and β-type as recently shown by Bygdeman (1968). Agrin MJ 1999 and Inpea exerted a weaker action while dihydroergotamine and some other α-blockers were strongly active. These results suggest that the uptake of NA in the platelets (presumably in their amine storage granules) occurs in a similar way in the serotonin-granules as in the specific NA-granules, and can be inhibited in the same way. It is also interesting to note that in the system used by Bygdeman, in which labelled NA was present in the relatively low concentrations 5×10^{-8} M—10^{-6} M, and thus exerted a lower degree of competition with the blockers on the uptake, these were still very active in 10^{-5} M concentration.

The possibility should also be considered that the granules might possess both α- and β-receptors involved in the binding of the transmitter. As shown in a previous study (Euler and Lishajko 1967b) not only NA but also A and isopropyl NA are readily taken up and retained by the granules. In this context it should also be recalled that the stimulating effect of catecholamines on heart adenyl cyclase is inhibited by α- as well as by β-blockers (Murad *et al.* 1962). It is thus conceivable that if two different systems co-operate in the binding process one of them may be associated with the release. Another observation which may be relevant in this connection, is that the β-blockers in general leave the NA release from granules unaffected or even enhance it in concentrations which strongly inhibit uptake while most α-blockers inhibit the NA release. If the release is a result of an active process normally mediated by α-receptors, the inhibitory effect on the NA release by α blockers may be explained. The uptake on the other hand may be primarily mediated by β-receptors although α-blockade may affect a common factor operating both in release and uptake.

It might be expected that efficient inhibitors of reuptake (like reserpine) should tend to cause partial emptying or depletion of the NA stores. This has been shown to occur for phenoxybenzamine (Schapiro 1958) and chlorpromazine (Johnson 1964) but has not been shown to be a rule in this series of compounds. We have tested the effect of one of the most active compounds, propranolol, in doses of 10—50 mg/kg in the guinea pig and found a moderate lowering of NA in the heart or kidneys in 6 hrs with the larger dose.

The present series of experiments has lent further support to our previously pre

sented hypothesis that adrenergic blockers of the α and β type react with some receptor mechanism in the adrenergic nerve granules which have properties in common with the receptors in effector cells.

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References

Ablad, B., M. Brogård and L. Ek, Pharmacological properties of H 56/28—a beta-adrenergic receptor antagonist. *Acta pharmacol.* (Kbh.) 1967. 25. Suppl. 2, 9—10.

Bygdeman, S., Effect of α- and β-receptor blocking agents on platelet aggregation and uptake of noradrenaline. *Acta physiol. scand.* 1968. 3 . 84—29A.

Euler, U. S. v. and F. Lishajko, Effect of some drugs on noradrenaline release from nerve granules. *Biochem. Pharmacol.* 1961. 8. No. 1

Euler, U. S. v. and F. Lishajko, Effect of adenine nucleotides on catecholamine release and uptake in isolated adrenergic nerve granules. *Acta physiol. scand.* 1963. 59. 454—461

Euler, U. S. and F. Lishajko, *Effect of drugs on the storage granules of adrenergic nerves.* In "Pharmacology of cholinergic and adrenergic transmission" Proc. 2nd Internat. Pharmacol. Meeting Prague 1963. Czechoslovak Medical Press, Praha 1965. 245—259

Euler, U. S. v. and F. Lishajko, Inhibitory action of adrenergic blocking agents on catecholamine release and uptake in isolated nerve granules. *Acta physiol. scand.* 1966. 68. 237—26-

Euler, U. S. v. and F. Lishajko, Reuptake and net uptake of noradrenaline in adrenergic nerve granules with a note on the affinity for l- and d-isomers. *Acta physiol. scand.* 1967a. 7 151—16-

Euler, U. S. v. and F. Lishajko, The uptake of isoprenaline in nerve granules. *Int. J. Neuropharmacol.* 1967 b. 6. 431—434

 U. S. v. and F. Lishajko, *Observations on the actions of prenylamine (Segontin®) on adrenergic transmitter granules.* Symposium on "Biochemical Aspects of Prenylamine" Capri 3—4 October 1967. In press 1968 a.

 U. S. and F. Lishajko, Effect of directly and indirectly acting sympathomimetic amines on adrenergic transmitter granules. *Acta physiol. scand.* 1968b. 73. 78—92

Euler, U. S. , L. Stjärne and F. Lishajko, Uptake of radioactively labeled dl-catecholamines in isolated adrenergic nerve granules with and without reserpine. *Life Sci.* 1963. 878—885

Gillespie, J. S. and S. M. Kirpekar, The inactivation of infused noradrenaline by the cat spleen. *J. Physiol.* Lond. 1965. 176. 205—227

Hertting, G., J. Axelrod and L. G. Whitby, Effect of drugs on the uptake and metabolism of H³-norepinephrine. *J. Pharmacol. exp. Ther.* 1961. 134. 146—153

Iversen, L. L., The inhibition of noradrenaline uptake by drugs. *Adv. Drug Res.* 1965. 5—23

Johnson, G. E., The influence of chlorpromazine on the catecholamine excretion of normal and cold acclimated rats. *Acta physiol. scand.* 1964. 60. 181—188.

Lindmar, R. and E. Muscholl, Die Wirkung von Pharmaka auf die Elimination von Noradrenalin aus der Perfusionsflüssigkeit und die Noradrenalinaufnahme in das isolierte Herz. *Arch. exp. Path. Pharmak.* 1964. 247. 469—492

Moran, N. C., Pharmacological characterization of adrenergic receptors. *Pharmacol. Rev.* 1966. 18. 503—51

Murad, F., Y. M. Chi, T. W. Rall and E. W. Sutherland, Adenyl Cyclase III. The effect of catecholamines and choline esters on the formation of adenosine 3',5'-phosphate by preparations from cardiac muscle and liver. *J. biol. Chem.* 196-. 237. 1233—1238

Schapiro, S., Effect of a catechol amine blocking agent (dibenamine) on organ content and urine excretion of noradrenaline and adrenaline. *Acta physiol. scand.* 1958. 4 . 3 —375.

Stjärne, L., Studies of catecholamine uptake, storage and release mechanisms. *Acta physiol. scand.* 1964. 62. Suppl. 228

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Fluorescence Microscopic Localization of Histamine in Canine Mastocytoma

By

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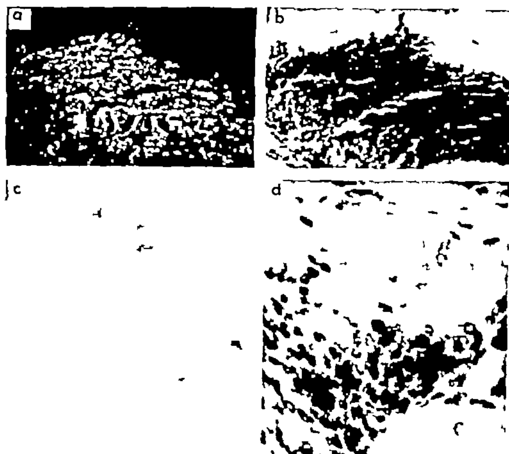
Histamine can be measured in tissues and body fluids through the formation of fluorophores with o-phthaldialdehyde (Shore *et al.* 1959) The reaction has been used in the development of histochemical techniques for histamine in tissue sections (Juhlin and Shelley 1966) and a method is now available which allows a precise localization of histamine not only in mast cells, but also in a large system of epithelial cells in the murine gastric mucosa (Ehinger and Thunberg 1967 Håkanson and Owman 1967 Ehinger *et al.* 1968) Studies are in progress to test the sensitivity specificity and applicability of this new histochemical technique. Dog mastocytoma was chosen as a suitable tissue model because of its high concentration of histamine (Cass *et al.* 1954)

Mastocytoma tumours were removed from various sites during operation (courtesy of Ass. Professor Stig Börnfors) upon 5 adult dogs of either sex. Shortly after removal the material was quenched to the temperature of liquid nitrogen, freeze-dried and embedded in paraffin *in vacuo* Sections were cut at 6 μ thickness, deparaffinized in xylene and treated in o-phthaldialdehyde vapours, followed by water exposure, all according to Ehinger *et al.* (1968) The sections were analyzed in fluorescence microscope with a HBO 200 high pressure mercury lamp and UG 1 activation and GG 9 barrier filters.

Fluorescence microscopy revealed the presence of a large number of intensely yellow fluorescent cells that were usually densely packed in groups (Fig 1a and b) or lay scattered in the connective tissue stroma some regions of the tumours were almost devoid of fluorescent cells. The cells were identified as mast cells through their metachromasia after staining with toluidine blue in ethanol solution. No other cells with a specific, o-phthaldialdehyde induced fluorescence were found in the tissues and comparison with the section when subsequently stained with toluidine blue showed that all metachromatic cells were fluorescent (Fig 1 and d)

Microspectrographic comparison (Björklund *et al.* 1968) between o-phthaldialdehyde treated dry microdroplets containing authentic histamine (100 μg/ml distilled water pH 7.5) and the fluorescent mast cells showed a good agreement in the appearance of the activation and emission curves (activation maximum 440 mμ. emission maximum 500 mμ uncorrected instrumental values)

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Dog mastocytoma. Section first treated in *o*-phthaldialdehyde vapour to demonstrate histamine (a and c) and subsequently stained with toluidine blue for identification of mast cells through their metachromasia (b and d). (a) Dense group of intensely yellow-fluorescent cells. (b) The entire group appears metachromatic upon toluidine blue staining. 70 × (c) Higher magnification of fluorescent cells in another region of the tumour. (d) Each fluorescent cell corresponds to one exhibiting metachromasia upon toluidine blue. Note that also the fluorescence intensity agrees with degree of staining. 125 ×

References

BJÖRKLUND, A., B. EHINGER and B. FALCK, A method for differentiating dopamine from noradrenaline by microspectrofluorometry. *J. Histochem. Cytochem.* 1968 I press.

CASS, R., J. F. RILEY, G. B. WEST, K. W. HEAD and S. W. STROUD, Heparin and histamine in mast-cell tumours from dogs. *Nat* [illegible] (Lond.) 1954 *174* 318—319.

EHINGER, B., R. HÅKANSON, CH. OWMAN and B. SPORRONG, Histochemical demonstration of *histamine in paraffin sections by fluorescence method. Bioch. Pharmacol.* 1968. In press.

EHINGER, B. and R. T[illegible], Induction of fluorescence in histamine-containing cells. *Exp. Cell Res.* 1967 *47* 116—122.

HÅKANSON, R. and CH. OWMAN, Concomitant histochemical demonstration of histamine and catecholamines in enterochromaffin-like cells of gastric mucosa. *Lif. Sci.* 1967 *6* 759—766.

JUHLIN, L. and W. B. SHELLEY, Detection of histamine by new fluorescent o-phthalaldehyde stain. *J. Histochem. Cytochem.* 1966. *14* 525—528.

SHORE, P. A., A. BURKHALTER and V. H. COHN, A method for the fluorimetric assay of histamine in tissues. *J. Pharmacol. Exp. Ther.* 1959 *127* 182—186.

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Analysis of the EEG of the Guinea Pig Fetus

by

Dag Stenberg

Intra-uterine guinea pig fetuses have been studied with a method modified from that of Bergström (1962). Only nitrous oxide inhalation and local anesthetics were used, and the mother animal was curarized and artificially respirated during the experiment. 75 EEG samples (neocortex, lower brain stem, and hippocampus) from 16 fetuses (gestational age 37 to 65 days) and from 2 new-born animals were analyzed with a μ-Linc computer. The power spectra were transformed from the autocorrelograms. Spectra from the same general region of the brain and the same age group were pooled and averaged (Fig. 1). In all of the material there is a dominance of very low frequencies, which is attributed partly to random slow artefacts, though also partly to slow EEG activity. The main change during development is a relative increase in theta range and alpha range activity (Fig. 2), especially in the hippocampus and least in the lower brain stem. Calculations of the statistical significance of the changes in theta intensity showed a significant increase at $p < 0.05$ between the second and third age groups in the cortical samples, and between the third and fourth age groups in the lower brain stem. The maximum changes might be related to the development of functional connections between different brain regions.

In the amplitude domain, the variance $\left[V = 1/T \int x^2(t)dt\right]$ of the EEG amplitude $[x(t)]$ and the coefficient of variation of the variance ($C = s_V/\bar{V}$, where s_V



Fig. 1. Averages from power spectra of cortical EEG (number of samples in the groups, 4, 11, 7 and 4 respectively). For clarity of display the ordinate values represent the square root values of the averaged spectrum.

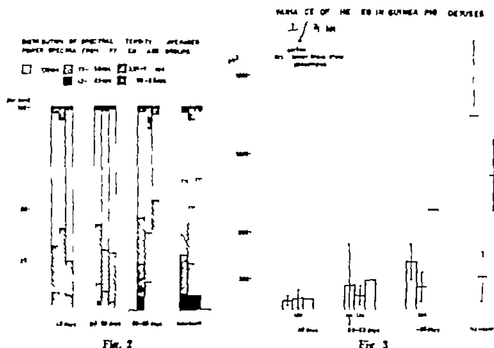


Fig. 2 Fig. 3

Fig. 2. The power spectral intensity in different frequency bands in the cortical samples. Cx = cortex, lbs = lower brain stem, hc = hippocampus.

" 3. The variance of the EEG and its standard deviation in different age groups and brain regions.

is the standard deviation of the variances of consecutive 5 sec samples during 1 min of EEG the total variance of which is V) were computed. The increase in variance (Fig 3) in the cortical recordings is significant at $p<0.05$ or better between all age groups except the second one In the two oldest groups the difference between cortex and lower brain stem is significant at $p<0.05$ The coefficient of variation of the variance decreased with age thus the amplitude of the EEG became less variable during development, although it was always sensitive to the general state of the fetus.

References

Bergström, R. M., A Surgical Technique for the Study of Brain, Muscle and EEG Potentials in the Foetus. *Ann. Chir. Gynaec. Fenn.* 1962 *51* 504—510.

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Surgical Denervation of the Cutaneous Blood Vessels

By

Göran Jurell, Karl-Axel Norberg and Björn Palmér

By an incision through the skin, sympathetically innervated structures, sweat glands and smooth muscle of the hair follicles and blood vessels on both sides of the incision become denervated. When forming a skin flap the same will be the result. The behaviour of the cutaneous vessels under various surgical conditions has been studied (Hynes 1951—52).

Skin flaps 2 times 5 cm large were formed on one side of the back of 30 rats (Sprague Dawley) elevated and then sutured in their former place. At various postoperative intervals biopsies were taken from different parts of the flaps and the control side. The biopsies were processed according to the formaldehyde method of Falck and Hillarp (for references, see Norberg 1967) sectioned horizontally or vertically to the surface and mounted for fluorescence microscopy. In some cases, stretch-preparations were performed of the fascia under the panniculus muscle, freeze-dried and formaldehyde treated (Fig. 1 and 2).

The blood flow through the flap was studied daily during the healing period by means of the ^{133}Xenon clearance method (see Larsen, Lindberg and Munck 1964). Intracutaneous injections of 0.05—0.1 ml of a solution of ^{133}Xenon in isotonic saline (Radiochemical Centre Amersham, England) were performed in various parts of the flap and control skin and the disappearance rate was recorded for the first 4 min. The values for the flaps were expressed in per cent of the control values.

The blood vessels in a flap were found to be denervated up to the base within 48 hrs after surgery (Fig. 3). Reinnervation of the blood vessels occurred about 8—16 weeks postoperatively (Fig. 5). From the first postoperative day the Xenon clearance was high in the base, sometimes even higher than that of the control side, which might be interpreted as a state of vasodilatation due to the denervation. The values for the apex of the flap, however, were very low the first postoperative day; during the first week slowly rising to about 70 per cent of those for the control side (Fig. 4).

The "supersensitivity" for monoamines in denervated structures has been described by many authors (see Trendelenburg 1963). Clinical observations (Klingenström, Nylén and Westermark 1966) have indicated that adrenaline as an adjuvans to local anesthesia will increase the area of necrosis in an experimental flap. This might be due to increased sensitivity of the denervated vessels, partly as a result of high concentrations of catecholamines at the effector cells occurring because of a lack of inactivation by uptake in the terminals.

The present work has provided means to study the behaviour of blood vessels histologically shown to be denervated. The blood flow through the base of a skin flap has been found to be similar to that of a control skin area immediately postoperatively, while the flow through the peripheral part of the flap is very low at that time, and then gradually increases. Experiments are in progress to study the influence on the blood flow of biogenic monoamines injected locally or administered

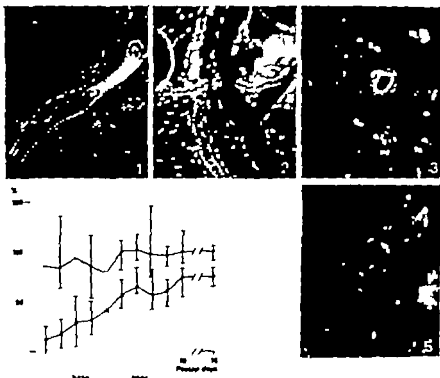


Fig 4

Fig 1 Subdermal artery longitudinal section, with normal adrenergic innervation, in the right part surface view of the adrenergic ground plexus. ×40.

Fig 2 Stretch-preparation of the submuscular fascia of the panniculus carnosus. Note the rich innervation of the arterioles (left) and absence of adrenergic nerves in the venules (right) ×60

Fig 3 Denervated subdermal vessels, transverse sections. ×60.

Fig 4 ¹³³Xenon clearance of skin flaps (base and apex) on the back of rats, in per cent of contralateral control values Mean values and range of 19 rats.

Fig 5 Beginning reinnervation in subdermal vessel of skin flap, 8 weeks postoperatively ×60.

parenterally in an attempt to elucidate some of the reasons for failure of the flap surgery technique

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References

Hynes, W The blood vessels in skin tubes and flaps. *Brit J pl st S g* 1951—5 . 3 165—1 5.
Klingenström P B. N and L Westermark, Vasoconstrictors and experimental flaps. *Act hir scand* 1966, *131* 18 —192
Lassen N A J Lindbjerg nd O M nck, Measurement of blood-flow through skeletal muscle by intramuscular injection of Xenon-133 *Lanc t* 1964 *1* 686—689
Norberg, K A., Transmitter histochemistry of the sympathetic adrenergic nervous system. *Bra R s* 1967 *5* 125—17
Trendelenburg, U Supersensitivity and subsensitivity to sympathomimetic amines. *Pharmacol. R* 1963, *15* 225—276

Acta physiol. scand. 1968. 74. 513—520

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Monoamine Oxidase in Sympathetic Ganglia of the Cat

By

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Abstract

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The monoamine oxidase (MAO) activity of sympathetic ganglia and isolated ganglion cells of the cat has been studied by micromodification of the radiochemical method of McCaman *et al.* (1965a) using (^{14}C) tyramine. The superior cervical, stellate and coeliac ganglia showed high MAO activity, the coeliac having almost twice the activity of the others. The 6th and 7th lumbar and the 1st sacral ganglia showed lower and approximately the same activity. No significant changes in MAO activity could be detected in the lumbo sacral ganglia three to four weeks after denervation.

The 7th lumbar ganglion was used for the single cell experiments. 75 per cent of the ganglion cells had measurable MAO activity with range from 1 to 41 moles of product $\times 10^{-13}$/hr (mean ± S.E. = 5.5 ± 0.57). The results support the previous suggestion that the sympathetic ganglia of the cat contain two distinct cell populations, firstly, cholinergic population, representing in L7 about 10—15 per cent of the ganglion cells characterized by the presence of ChAc, high concentrations of AChE and the absence of monoamine fluorescence and MAO activity, secondly an adrenergic population, about 75—88 per cent of the ganglion cells, which exhibits fluorescence for noradrenaline and MAO activity, contains low or moderate AcChE and no measurable ChAc activity.

Previous histochemical and biochemical studies indicate that the sympathetic ganglia of the cat contain at least two functionally distinct cell populations (Sjöqvist 1962, Giacobini *et al.* 1967, Buckley *et al.* 1967b). These populations may be defined biochemically in terms of the transmitters and the related enzymes present in the individual neurons. The activity of these enzymes can be studied quantitatively by combining micromanometric and microisotopic methods (Giacobini *et al.* 1967, Buckley *et al.* 1967a, Buckley *et al.* 1967b).

Although monoamine oxidase (MAO) plays an important role in the inactivation of monoamines there is uncertainty about its exact localization in nervous structures. This is due to the fact that no sensitive and precise methods for demonstrating this enzyme have been available. In the present paper the MAO activity of isolated

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sympathetic ganglion cells has been studied by a modification on a microscale of the isotopic method of McCaman *et al* (1965a) Normal and preganglionically de nervated 7th lumbar ganglia of the cat have been used.

Material and methods

Dissectio and denervatio procedure

Adult male and female cats were anesthetized i.p. with sodium pentobarbitone (Nembutal, Abbott, 40 mg/kg) The lumbosacral ganglia were freed and either removed for immediate biochemical analysis, or denervated as follows. The sympathetic chain was removed bilaterally between L_4 and L_5 for length of about 2 cm. The sympathetic trunk was then cut between L_4 and L_5. The operation was performed under aseptic conditions as previously described (Holmstedt, Lundgren and Sjöqvist 1963, Buckley *et al.* 1967b) Excessive bleeding did not occur during the operations, nd ery little tissue reaction around the sympathetic chain was evident t the subsequent dissection of the ganglia 2—7 weeks later

After removing the connective tissue capsule the dissected ganglia were either weighed, homogenized and analyzed for total MAO activity or placed in physiological saline for preparation of individual ganglion cells nder the dissection microscope. In the latter case individual cell bodies were rapidly isolated by means of gentle manipulation using sharp needles, and sucked into a micropipette having olume of about 0.5 µl as used for a Cartesian diver (Giacobini 1957) The cells were then transferred to small glass tube as previously described (Buckley *et al* 1967a) *Each preparation consisted of one cell body approximately of* the same size (±10 %) together with the initial segment of the axon, approximately 50 µ long (Giacobini 1957) The olume of Ringer solution or saline carried with the cells as less than 0.1 µl. The presence of the cell preparation in the bottom of the tube was checked under the microscope. After the addition of the buffer-substrate or buffer-substrate-detergent, the histological features of the cell bodies were no longer learly discernible.

Monoam idase determinatio

Monoamine oxidase activity was determined by the following adaptation of the method of McCaman *et al* (1965a) using (^{14}C) tyramine the modification permitted the measurement MAO activity of isolated cells, in p to 150 individual cells in one experiment.

Triton X 100 (iso-oct l-phenoxy-poly-etoxyetanol) a powerf l detergent, was added in some experiments in order to disrupt the cell membranes. It was dissolved in distilled ater and used at the concentration indicated (see Fig 1)

a) Reagents

Buffer substrate BS was prepared fresh for each experiment to give the final concentration indicated

16.85 µl of ^{14}C ramine New England Nuclear Corp., 5 mC/mM) was diluted to 10.8 µl phosphate buffer 0.2 M pH 7.8 In some experiments an equal olume of Triton X 100 was added giving final concentration of (^{14}C) tyramine which aried between 2.56 and 3.03 mM

b) Procedure

1 The individual ell preparation was added to 1 µl BS and incubated for 1—2 hrs at 38 C, the final concentration of ^{14}C tyramine was 3.03 mM. To prevent evaporation the tubes were either sealed h rubber caps or 50 µl hexane was pipetted onto the BS

2. The reaction was stopped with 10.8 µl 0.75 N HCl, and 54 µl of ethyl acetat as added. The seals were previously removed

3. The preparation was mixed thoroughly "buzzed" and centrifuged for 5—10 min at 4000 rpm and 2 C

4 44 µl of the ethylaceta supernatant was washed with 29.1 µl 0.4 N HCl

5 This was shaken thoroughly and centrifuged for further 10 min t 4000 rpm and 2 C

6. 31.4 µl of the supernatant was placed in the counting ial, 0.75 µl Hyamine as 0.3 M solution in methanol and 10 ml PPO POPOP were added (4 g PPO 0.1 g POPOP in one litre of toluene)

7 The radioactivity was measured Packard liquid scintillation spectrometer

All operations except incubation were arried out t 2 C. The ganglion homogenates (2—4 µg/µl) were incubated in 10 µl olumes for 15—30 min at 38 C. Tubes containing BS without tissue were run through the whole procedure and used as blanks. The cells were

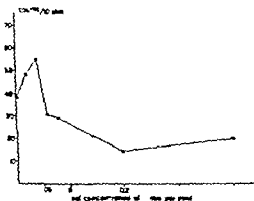


Fig 1

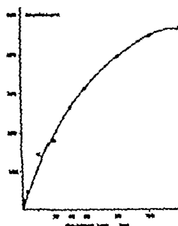


Fig 2

Fig. 1 Effect of different concentrations of Triton X 100 on the MAO activity of rat brain homogenate (5 µg/µl)

Fig. 2. Time course of the oxidation of (¹⁴C) tyramine by rat brain homogenate (40 µg/µl)

incubated for 1—2 hrs because it was found that over that time period the products of the reaction (p-hydroxyphenylacet acid and p-hydroxyphenylacetaldehyde) increased propor tionally with time and enough product was accumulated for measurement

Controls

Homogenates of rat brain er convenient source of enzyme for control experiments. These were prepared at oncentration of 40 µg of fresh brain per µl H_2O in glass homogenizer Further dilutions of the homogenate were made th 0.05 per cent bovine albumin or with the BS. The pipetting of small volumes of the homogenates as facilitated by using the super natant after centrifuging at 2000 rpm for 10 min. This step may however produce some loss of enzyme activity The preparation of the homogenates and all manipulations ere performed at 0—2 C.

Three types of controls wer used buffer plus homogenate which had been boiled for 30 min b) tissue plus buffer ithout ¹⁴C tyramine buffer withou ssue Ther ere no significant differences in the control alues, so 0 1 µl buffer substrate how ever as used routinel as control

The tubes ere sealed with rubber caps to preven evaporation er eturned to he ice and the caps emoved after incuba ion at 38 C.

The relationship betw ... incubation ... ... the effect of detergent

The relationship between the metabolism of ¹⁴C tyramine and enzyme concen ion as examined and it as show over at least an 60-fold range he ... of tyramine as proportional to the quantit of enzyme The time ourse of he ... of ¹⁴C tyramine by 40 µg/µl of rat br in homogenates during different ... hi ... ela tionship during the first 60 min Fig 2

The detergent Triton X-100 was added homogena es ... ions of Triton from 0.01 ... I these experimen ... he best ac tivation Fig. 1 On the other hand no significa ... of activ cells as found in the presenc and absenc of T ... of activ observed

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TABLE I

Type of ganglion	MAO activity*		Catecholamines	
	Normal	Denervated	A	NA
Superior cervical	6.5 ± 0.3 (5)	—	0.12	8.7
Stellat	6.6 ± 0.8 (6)	—	0.13	9 4
L_6	4.2 ± 0 4 (11)	4 1 ± 0 4 (7)		
L_7	4.2 ± 0.3 (13)	4 1 ± 1 1 (5)	0.80	6.8
S_1	4 0 ± a.3 (13)	4.3 ± 0 4 (6)		
Coelia	11 1 ± 0.6 (6)	—	3.3	17 7
Inferior mesentericum	12 1 (2)	—	9 6	24 7

MAO activity expressed in moles of product 10^{-4} /hr/µg wet

K. A. Norberg and F Sjoqvist, Pharmacol. Rev 1966. *18*, 743—751

Means of 3 expts. The data are expressed in µg/g.

= adrenaline

= noradrenal ne

mean + K × S.D

were K is constant obtained from "Documenta Geigy Scientific tables

With tolerance probability of 99 per cent, confidence probability of 95 per cent for N=103 (Table II) the constant K = 2 9 In this case t least 99.5 per cent of the distribution is within the tolerance limit.

A distribution curv constructed for the controls and the cell counts showed tha there were tw populations of cells The majority of the cells ga counts outside the ange of the controls but some cells were inside this range These former cells were also outside the range of three times the standard deviation of the controls It was concluded that these cells ere activ and the remainder possessed MAO activity less than 10 * moles of product/hr

Results

The MAO acti ities of normal and denervated ganglia were the same and re given in Table I

The sympathetic ganglia studied show three different levels of MAO activity L_6, L_7 and S_1 show the lowest activ ty the coeliac and the inferior mesenteric the highest activity while the superior erv cal and the stellate are intermediate There is a good correlation between the MAO activ ties and the catecholamine co tents particularly noradrenaline (NA)

TABLE II Distribution of cells with and without MAO activity in 7th lumbar ganglia of the cat.

Normal ganglion cells				
Exp. no.	Active	Inactive	Total	Per cent active
I	33	13	46	72.0
II	37	10	47	78.5
III	33	16	49	67.5
n = 3	103 (73 %)	39	142	M = 72.6

The results reported in Fig. 3 and Table II represent experiments in 3 cats and 142 isolated cell bodies from the 7th lumbar ganglion. 103 cells showed measurable MAO activity between 1.0 to 41.0 moles of product $\times 10^{-12}$/hr. These cells represented 73 per cent of the population investigated and their mean activity was 5.5 ± 0.57 (mean $\pm$ S.E.). The majority had low MAO activity while a few cell bodies had very high activity above 20 moles of product $\times 10$ /hr (Fig. 3). The percentage active cells in each experiment varied (Table II) between 67.5 and 78.5.

Discussion

In the present study a scaled-down modification of the method described by McCaman *et al.* (1965a) was used in order to permit the determination of MAO activity in ganglia and in single nerve cells weighing about 0.001 µg dry weight. Comparing the MAO activities of some sympathetic ganglia the stellate and superior cervical ganglia showed about half the activity of the coeliac ganglion (Table I).

The MAO activity in the 7th lumbar ganglion was not changed after preganglionic denervation. By contrast Giacobini *et al.* (1967) and Buckley *et al.* (1967b) found a marked decrease in acetylcholinesterase (AcChE) and cholineacetylase (ChAc) activity in this ganglion after denervation. The latter is in agreement with the behaviour of ACh in the same ganglion (Friesen *et al.* 1967).

Comparing the relative activities of AcChE, ChAc and MAO in some autonomic ganglia of the cat, it becomes evident that an excellent correlation exists between AcChE and ChAc activities (Table III). With respect to their ChAc and AcChE activity the ganglia can be ranked in the following progressive order: coeliac, L_6, S_1, L_7, stellate, superior cervical and ciliary. The lowest level of ChAc and AcChE is found in the coeliac ganglion, which exhibits the highest MAO activity. Inversely the ciliary ganglion, which shows the highest ChAc and AcChE activity, has the lowest MAO activity (about 1/2 of L

Interestingly the coeliac and inferior mesenteric ganglia have an abundant system of adrenergic synaptic terminals (Norberg and Sjöqvist 1966) while the ciliary ganglion contains only a few synaptic adrenergic structures (Hamberger *et al.*



Fig 3

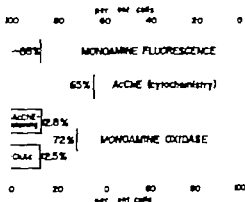


Fig 4

Fig. 3 Frequency distribution of MAO activity—in moles of product ×10⁻¹⁴/hr/cell in normal sympathetic ganglion cells of the cat 7th lumbar ganglion—

Fig. 4 Diagram of the percentage of the cells from the 7th lumbar ganglion of the cat containing AcChE, ChAc, monoamines and MAO The figure is based on data from Buckley *et al.* 1967b, Giacobini *et al* 1967 Hamberger *et al.* 1965a, Sjöqvist 1962 and the present investigation.

1965b) The nodose and thoracic spinal ganglia show rather low MAO activity (Kerpel-Fronius and Giacobini 1968, to be published)

Our experiments with isolated cell bodies show that approximately 70 per cent of the perikarya in L_7 contain the enzyme required for the oxidation of monoamines (Table II) This figure agrees relatively well with the percentage of cells (88 %)) in which monoamine fluorescence can be demonstrated (Hamberger *et al* 1965a, Fig. 4) It should be stressed that the percentage of active cells were the same with or without triton an agent known to disrupt cell membranes. Hence it seems unlikely that the number of active cells should depend on the accessibility of the polar substrate to the enzyme

It has been previously reported (Giacobini *et al.* 1967 Buckley *et al.* 1967b) that in L_7 *the number of ganglion cells* containing AcChE *(65 %) considerably exceeds* the 12 per cent of cells containing measurable ChAc and lacking monoamine fluorescence (Fig 4) This indicates that AcChE is located not only in cholinergic neurons but in monoamine neurons as well (*cf* Giacobini *et al.* 1967) Our results suggest that in L the proportion of cells storing monoamines is roughly the same as the number of cells containing measurable MAO Hence MAO may be more specifically distributed than previously believed on the basis of histochemical studies (Koelle 1951 Koelle and Valk 1954

In summary our results are compatible with the view that the sympathetic ganglia of the cat contain two distinct cell populations (Fig 4) firstly a "cholinergic" population, representing in L about 10—15 per cent of the ganglion cells,

Table III Cholinacetylase, acetylcholinesterase and MAO activity in various autonomic ganglia of the cat.

Ganglion	S_1	L_7	L_4	Stellate	Coeliac	Superior cervical	Ciliary
ChAc	100 ± 0.9 (5)	100 ± 0.5 (5)	88 ± 0.9 (5)	120 ± 10.6 (4)	48 ± 3.2 (5)	175 ± 6.6 (6)	242 ± 18 (5)
AcChE	8.42 ± 0.54 (7)	8.70 ± 0.78 (10)	5.35 ± 0.22 (10)	8.91 ± 0.97 (4)	4.46 ± 0.41 (8)	9.21 ± 0.30 (4)	15* (5)
MAO*	4.00 ± 0.3 (13)	4.20 ± 0.5 (15)	4.20 ± 0.4 (11)	6.60 ± 0.8 (6)	11.1 ± 0.6 (6)	6.50 ± 0.3 (5)	1.9* (2

Buckley *et al.*, Acta physiol. scand. 1967 *71* 348—356.
ChAc activity expressed in moles ACh × 10^{-10}/hour/µg wet.
The values represent means ± S.E. Number of cats within parenthesis.
Holmstedt *et al.* Acta physiol. scand. 1963. 57 235—246.
AcChE activity expressed in µmoles HAc × 10^{-4} min./mg.
The values represent means ± S.E. Number of cats within parenthesis.
Sjöqvist, F 1962. Same units as in *)
MAO activity expressed in moles of product × 10^{-11} hour µg wet.
The values represents means ± S.E. Number of cats within parenthesis.
Kerpel-Fronius, S and E. Giacobini, 1968. T be published.

characterized by high concentrations of AcChE and the absence of monoamine fluorescence (Hamberger *et al.* 1965a) These cells ma be identical with those containing ChAc (Buckley *et al.* 1967b) and may also belong to the group of neurons lacking measurable MAO The remaining neurons, the adrenergic ganglion cells exhibit fluorescence for noradrenaline (Hamberger *et al* 1965a and contain low or not measurable AcChE. These neurons may be identical with those containing MAO

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References

Buckley G S Consolo E Giacobini and R M [illegible] de-
termination of choline acetylase in individual [illegible] 34 [illegible]
Buckley G S Consolo E Giacobini and F [illegible]
denervated sympathetic ganglia and ganglic [illegible]
71 348—356
Documenta Geigy Scientific Tables Sixth Edition Ba[illegible]

FRIESEN, A., G. M. LING and M. NAGAI, Choline and phospholipid-choline in sympathetic ganglion and their relationship to acetylcholine synthesis. Nat. (Lond.) 1967 214 722—724

GIACOBINI, E. Quantitative determination of cholinesterase in individual sympathetic cells. J. Neurochem 1957 1 234—244

GIACOBINI, E., B. PALMBORG and F. SJÖQVIST. Cholinesterase activity in innervated and denervated sympathetic ganglion cells of the cat. Acta physiol. sc nd. 1967 69 355—361

HAMBERGER, B., K. A. NORBERG and F. SJÖQVIST. I Pharmacology of cholinergic and adrenergic transmission. Ed. by G. B. Koelle, W. W. Douglas and A. Carlsson. Proc. 2 d I Pharmacol M t (Prague) 1963a. 3 41—53

HAMBERGER, B. K. A. NORBERG and U. UNGERSTEDT. Adrenergic synaptic terminals in autonomic ganglia. Act physiol. scand 1965b. 64 285—286.

HOLMSTEDT, B., G. LUNDGREN and F. SJÖQVIST. Determination of acetylcholinesterase activity in normal and denervated sympathetic ganglia of the cat. A biochemical and histochemical comparison. Act phy iol. sc d 1963 57 235—247

KERPEL FRONIUS, S. and E. GIACOBINI, 1968. To be published.

KOELLE, G. B. The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and survey of their cellular distributions. J. Pharma ol. 1951 103 153—171

KOELLE, G. B. and A. T. VALK, Physiological implications of the histochemical localization of monoamine oxidase. J. Physiol. (Lond.) 1954 126 No. 3. 434

MCCAMAN, R. E., M. W. MCCAMAN, J. M. HUNT and M. S. SMITH, Microdetermination of monoamine oxidase and 5-hydroxytrypthophan decarboxylase activities in nervous tissues. J. Neurochem 1963a. 12 15—23

MCCAMAN, R. E., G. RODRIGUEZ DE LORES ARNAIZ and E. DE ROBERTIS, Species differences in subcellular distribution of choline acetylase in CNS. J. Neurochem 1965b. 12 927—935.

NORBERG, K. A. and F. SJÖQVIST. D New possibilities for adrenergic modulation of ganglionic transmission. Pharmacol Rev 1966. 18 743—751

SAUNDERS, L. and R. FLEMING, Mathematics and Statistics. The Pharmaceutical Press (London) 1957 p. 138

SJÖQVIST, F. Cholinergic sympathetic ganglion. Th. Thesis. Karolinska Institutet Stockholm. 1962

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Migration of Acetic Acid and Sodium Acetate and Their Effects on the Gastric Transmucosal Ion Exchange

By

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Abstract

Flemström, G. and B. Frenning. *Migration of acetic acid and sodium acetate and their effects on the gastric transmucosal ion exchange.* Acta physiol. scand. 1968. 74. 521—532.

The kinetics of the disappearance of acetic acid and sodium acetate from the stomach during secretion and secretory arrest was studied in anesthetized cats. Model experiments were also performed in which the solutes diffused through a cellophane membrane. On instillation of acetic acid into non-secreting stomachs, the hydrogen ion concentration decreased significantly more rapidly than the acetate concentration ($p<0.05$). The increase in the combined sodium and potassium ion concentrations was significantly greater than the increase in the chloride ion concentration. Thus, in addition to molecular diffusion of acetic acid, exchange diffusion took place between hydrogen ions and sodium ions. A small amount of secretion masked this exchange. The acetate disappeared more rapidly on instillation of acetic acid than on instillation of sodium acetate. Acetic acid increases the ionic permeability of the gastric mucosa and has inhibitory effect on secretion. It has been reported that this secretory inhibition persists for up to 24 hrs. By repeated instillation experiments in the cat it was found that the permeability effects of a 30 min installation of 170 mM acetic acid did not persist for longer than 90 min. Sodium acetate per se probably had no effect on the ionic permeability of the gastric mucosa. It is suggested that the permeability increasing effect of acetic acid is related to transient intracellular accumulation of the diffusing acid. Epithelial abrasion that could give rise to an increased permeability by decreasing the diffusion distance requires restoration of the surface epithelium within 90 min and in fact no abrasion was observed as an affect on acetic acid on the mucosa.

The permeability of the gastric mucosa to different substances has attracted great attention because of its important pharmacological aspects. Teorell (1939) found that on instillation into non-secreting cat stomachs weak acids disappeared more rapidly than strong acids. He suggested that weak acids in an undissociated and thus in a more lipid soluble form could permeate more rapidly through the non-aqueous part of the cell membrane. Schanker *et al.* 1957 showed that weak electrolytes rapidly diffused out of the rat stomach if the stomach pH was such that the electrolytes were undissociated. It has been suggested by Martin (1963) that a weak acid which diffuses in an undissociated form from the acid environment in

the stomach lumen accumulates intracellularly in the mucosal cells, where the pH is higher

It has been found that several weak acids increase the permeability of the gastric mucosa to ions. Davenport (1964) found that acetic acid increased the net flow of water hydrogen ions, sodium ions, potassium ions and chloride ions through the mucosa. He obtained a similar effect with propinic, butyric and acetylsalicylic acids. Solutions of the same acids buffered with TRIS and glycine to pH of about 7 had no effect on the flows. Flemström, Frenning and Öbrink (1964) showed that after instillation of 170 mM acetic acid the permeability coefficient for hydrogen ions on subsequent instillation of hydrochloric acid was increased by 100 %

Babkin, Hebb and Kreuger (1941) studied the effect of acetic acid instillation in dogs with an oesophagostomy and gastric fistula. The stomach was stimulated to secretion by sham-feeding or subcutaneous injection of histamine. After instillation of 1 % (i.e. 167 mM) acetic acid, there was a reduced secretion volume lower acidity and a reduced peptic power" The acidity value regained the normal level after 24 hrs. while the volume remained lower for several days. Grant (1944 1945) demonstrated epithelial abrasion after instillation of acetic acid, but she also found that several ordinary food substances had a similar effect.

In view of these results it seemed of interest to study in detail the kinetics of the disappearance of acetic acid and sodium acetate after their instillation into the stomach and to investigate the duration of their effect on the permeability of the mucosa to ions.

Methods

Experimental animals

The experiments were performed on cats weighing between 1.5 and 3.8 kg. The cats were starved for at least 18 hrs. before the experiments in order to produce non-secretory state. They were anesthetized initially with chloroform and anesthesia was then maintained by mixture of chloralose and urethane (1 10) given A midline incision was made and the stomach isolated by means of ligature at the cardia and pylorus, care being taken not disturb the blood supply to the stomach. A glass tube was then inserted into the pyloric end of the stomach, after which the abdomen was closed. The secretory state of the stomach was subsequently checked over period of about 2 hrs or more. The stomach was considered to be in state of secretory arrest when during period of 1 hr before the start of the experiment the amount of secretion did not exceed 0.4 ml and the acidity 10 mM. Some experiments were also performed on stomachs which were secreting spontaneously.

Analyses

Acidity determination. 0.05 ml samples were pipetted to 4 ml double distilled water and titrated with 0.01 M NaOH (indicator bromthymolblue). The coefficient of variation was ±1 % at 100 mM.

Chloride was determined electrometrically with silver-silver chloride electrodes on the same samples as were used for the acidity determination. 0.005 M $AgNO_3$ was used for titration. The coefficient of variation was ±0.5 % at 100 mM.

Sodium and potassium were determined flamephotometrically (flame photometer Eppendorf, Netheler and Hinz GMBH, Hamburg) after dilution of 0.05 ml samples in double distilled water. A propane-air flame was used. The coefficient of variation was ±2 % at 100 mM (for sodium).

14C determination. ^{14}C-labelled solutions of acetate and acetic acid were used. For analysis ^{14}C was converted to $^{14}CO_2$ by combustion method described by Kalberer and

Fig. 1

Fig. 2

Fig. 1. The activity in samples from the same ^{14}C-labelled acetic acid solution, which had been burnt according to Kalberer and Rutschmann on paper pretreated with NaOH of different strengths. The figure shows the activity as a function of the strength of the NaOH solution used for pretreatment of the paper.

Fig. 2. The activity in samples from the same ^{14}C-labelled acetic acid solution, which had been burnt after different drying times. The upper curve shows the result when paper prepared with 340 mM NaOH was used. The lower curve shows the result with unprepared paper.

Rutschmann (1961) and counted by liquid scintillation (Packard Tri-Carb 314 EX). In order to avoid evaporation of the volatile acetic acid, combustion paper (Filter paper No. 589 Schleicher and Schnell AG, Feldmeilen, Zürich, Switzerland), which had been previously soaked in NaOH solution and allowed to dry, was used. The CO_2 was absorbed in 15 ml of solution made by mixing 120 ml distilled 2-aminoethanol and 880 ml methanol. 10 ml of this solution was then mixed with 10 ml scintillation liquid (toluene containing 4.0 g/L PPO (2,5-diphenyloxazol) and 0.1 g/L POPOP (1,4-di-2-(5-phenyloxazolyl)-benzene)). The optimal NaOH concentration for pretreatment of the combustion paper was determined by burning samples from [illegible] 170 mM, ^{14}C-labelled acetic acid solution on paper which had been pretreated with NaOH of different strengths. The results are given in Fig. 1. The counting rate showed [illegible] plateau above approximately 0.17 M NaOH and 0.34 M NaOH was selected. A comparison was also made of the effect of the length of drying time on the radioactivity of samples when prepared and unprepared paper was used. These results are shown in Fig. 2, which gives the number of cpm as a function of the drying time. With prepared paper the counting rate was constant for drying times varying between 20 min and 24 h. With unprepared paper [illegible] considerable loss of activity was obtained. The samples were burned on prepared paper after drying at room temperature for 10–20 hr. The quantity loaded on the paper was 0.05 ml and the coefficient of variation for the counting rate was [illegible] 1.5 %, the expected statistical error for the same number of impulses due to the randomly distributed disintegrations being 0.3 %. The stability of the counter was tested by counting a ^{14}C sample 50 times during a period of about 10 hrs. The coefficient of variation was 0.63 %, expected statistical error 0.5 % [illegible] the stability of the apparatus [illegible] good.

General [illegible] of proc[illegible]

6 ml of the test solution was instilled [illegible] the stomach [illegible] experiment. [illegible] fifteen h[illegible] min the fluid in the stomach was d[illegible] ned [illegible] of the [illegible] ed and [illegible] ple [illegible] aken for analyses. A total of 0.1 ml was used for det[illegible] mina[illegible] of acid [illegible] chloride, sodium and potassium. A further 0.05 ml was used for aceta[illegible] det[illegible] [illegible] which took 3–4 min, the fluid remaining was instilled [illegible]

The experiments [illegible] the [illegible] chloride [illegible] or four 15 min periods [illegible] the same solution was 30 or 60 min respectively) except [illegible] some experime[illegible] [illegible] instilled in stomachs during "spontaneous secretion" [illegible] 30 or [illegible] M [illegible] acid [illegible] sodium

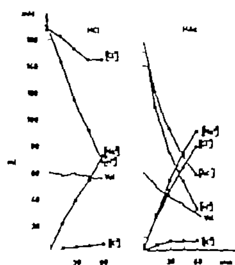
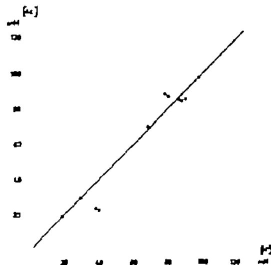



Fig 3 Fig 4

Fig 3 T the left, the resul of installation of 170 mM hydrochloric acid into a non-secreting cat stomach. T the right, the result of installation of 170 mM acetic acid into the same stomach.

Fig. 4 The acetate concentration plotted against the hydrogen ion concentration in the same sample The circles represent alues from installations in spontaneously secreting stomachs and the dots values from instillations into non-secreting stomach. The identity line Ac = H is drawn in.

etate solution was then given. T study the duration of the effect of acetic acid on the stric mucosa, three 30-min instillations of hydrochloric acid were given after the instillation acetic acid. This latter type of experiment was also performed with sodium acetate I ontrol experiments 5 consecutive 30-min instillations of 170 mM hydrochloric acid were given.

Model perim t

I ml of the test solution (170 mM HCl, HAc or NaAc) were placed in one chamber of diffusion cell separated from 4000 ml 170 mM NaCl by cellophane membrane. The surface of the membrane was 16 5 cm² Samples were taken from the test sol tion every 15th min for 1 hr Both compartments were tirred continuously

Results

D ffusion of ac ti acid and sodium a tate

6 ml of 170 mM hydrochloric acid was first instilled into a non-secreting stomach. One hr later the tomach contents were removed and 6 ml of 170 mM acetic acid or sodium acetat sol tion were instilled and analyses made over a similar time period In some experiments acetic acid was instilled into stomachs which exhibited spontaneous" secretion. The res lt are shown in Fig 3 4 and 5 and in Table I and II

The reduction in hydrogen ion ncentration was greater during instillation of acetic acid than during instillati n f hydrochloric acid into non-secreting stomachs (see Fig 3) There was a considerable decrease in ol me of the gastric contents

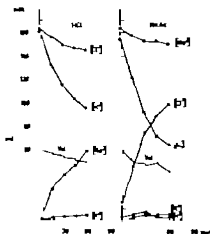


Fig 5

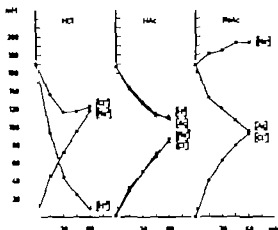


Fig 6

Fig. 5. Instillation of 170 mM hydrochloric acid and of sodium acetat solution in the same non-secreting cat stomach.

Fig. 6. Model experiment in which 12 ml 170 mM test solution was dialysed against 4000 ml 170 mM sodium chloride solution through cellophane membrane

during instillation of acetic acid this was probably due to the hypotonicity of the solution. The hydrogen ion concentration declined more rapidly than that of acetate. The combined sodium and potassium influx into the stomach was more rapid than that of chloride. The difference between the reduction in hydrogen ion concentration and the reduction in acetate concentration after 60 min was on the average, 14 mM. The corresponding difference between the combined increase in sodium ion and potassium ion concentration and the increase in chloride ion concentration was 9 mM. These differences were statistically significant ($p<0.05$ in both cases)

On instillation of cetic acid into pontaneously" secreting stomachs the acetate concentration decreased more rapidly than that of the hydrogen ions. The chloride ion concentration increased more quickly than the combined sodium ion and potassium ion concentrations. In other words the situation was th reverse of that observed on instillation into non-secreting stomachs. Thus even a very low secretion low secretion rate will tend to diminish the differences between ΔH and ΔAc and between Δ(Na+K) and ΔCl observed in non-secreting stomachs (see Table I) (For a general discussion on the influence of hydrochloric acid secretion on the appearent hydrogen ion permeability see Obrink and Waller 1965)

The results obtained from instillations f acetic acid into secreting and non-secreting stomachs are presented in Fig 4

Fig. 5 shows the results f typical xperiment th instillation of 170 mM sodium acetate solution into a non-secreting stomach. The sodium concentration decreased ery slowly while the increase in potassium concentration was of the same order of size as with acetic acid. The hydrogen ion concentration ncreased on the a erage

TABLE I Changes in composition of the test solution on instillation of 6 ml 170 meq/l acetic acid into the cat stomach during 1 hr

Secretory condition	ΔH mM	ΔAc mM	Δ(H−Ac) mM	ΔN mM	ΔK mM	ΔCl mM	Δ(N+K−Cl) mM	Δ ml
	−150	−133	−17	+104	+6	+99	+11	−2.1
	−153	−141	−12	+99	+5	+92	+12	−0.5
	−160	−130	−30	+112	+5	+98	+19	−2.6
Arrest	−145	−137	−8	+95	+4	+91	+8	−1.5
	−152	−153	+1	+95	+6	+104	−3	−1.3
	−137	−111	−26	+84	+7	+78	+11	−3.1
	−158	−155	−3	+93	+6	+98	+1	−0.7
Mean	−151	−137	−14	+97	+6	+94	+9	−1.7
	−124	−144	+20	+76	+7	+101	−17	−3.5
Spontaneous secretion	−122	−142	+20	+74	+9	+110	−27	+3.8
	−142	−155	+13	+101	+8	+126	−11	+1.4
	−146	−153	+7	+109	+6	+115	−2	−1.2
Mean	−134	−149	+15	+90	+8	+113	−15	2.5

by 3 mM (n=3). The acetate concentration decreased relatively rapidly but more slowly than when acetic acid was instilled. The chloride ion concentration increased rapidly. There was a mean volume decrease in the gastric contents of 1.2 ml.

Model experiment

The results are presented in Fig. 6 and Table II.

Model experiments with 170 mM hydrochloric acid gave essentially the same result as instillations into cat stomachs. In experiments with 170 mM acetic acid the decrease in hydrogen ion concentrations was smaller than in experiments with hydrochloric acid. The reduction in hydrogen ion concentration was equal to the reduction in acetate concentration. The increase in sodium ion concentration equalled the increase in chloride ion concentration.

TABLE II Model experiments in which 12 ml 170 mM inner solution was dialysed against 400 ml 150 mM NaCl across a cellophane membrane for one hr. The table gives the changes in concentration in the inner solution. mM ± S.E.

Inner solution	ΔH mM	ΔCl mM	ΔNa mM	ΔA mM	
HCl	−160 ± 2	−43 ± 2	+113 ± 2	—	7
HAc	−81 ± 4	92 ± 3	+93 ± 4	−78 ± 4	5
NaAc	—	96 ± 2	+21 ± 2	−70 ± 3	5

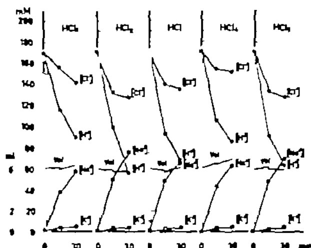


Fig. 7 Result of typical experiment with one hydrochloric acid installation (HCl) *before* and three consecutive hydrochloric acid installations (HCl_2—HCl *after* a 30 min instillation of 170 mM acetic acid. In this experiment one further acetic acid instillation followed by hydrochloric acid instillation (HCl_3) was given.

In experiments with sodium acetate the decrease in acetate concentration was of the same order of size as in experiments with acetic acid. The sodium concentration increased, probably because the chloride ion concentration increased more rapidly than the acetate concentration decreased, *i.e.* there was a diffusion effect (*cf* Teorell 1935 and 1937

The effects of cetic acid and sodium acet t th onic permeability f th gastric mucosa and their duration

170 mM hydrochloric acid was first instilled into a non-secreting cat tomach. Thirty min later the gastric contents were removed and were replaced by a similar volume of 170 mM acetic acid or sodium acetate solution. Finally repeated hydrochloric acid instillations were given. When changing the instillation solution th stomach was emptied as completely as possible The results are shown in Fig 7 and 8, and Table III and IV

After a previous instillation of acetic acid the eduction in hydrogen ion concentration on instillation of hydrochloric acid was greater than if not preceded by acetic acid. The sodium concentration increased more than before the acetic acid instillation, and the decrease in hloride ion concentration was greater The increase in potassium concentration was unchanged.

With subsequent hydrochloric cid instillations the changes in concentration of all ions except the potassium decreased The a erage concentration changes approached those observed during the initial hydrochloric acid in tillation. The olume changes were of the same order f magnitud throughout In a few experiments the instillation solutions darkened somewhat n evidenc of minor bleeding This did, however only happen d ring the las tw h drochloric acid instillations.

In the experiment ill ustrated in Fig f rther acetic acid instillation was given

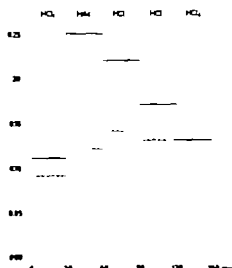


Fig 8

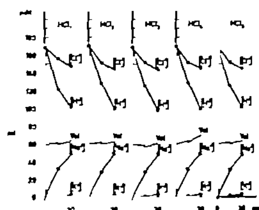


Fig. 9

Fig. 8. The mean permeability coefficients for hydrogen ions and sodium ions from eight experiments of the same type as in Fig. 7. The coefficients for sodium ions are shown by the broken lines.

Fig. 9. Result of an experiment with repeated instillations of 170 mM hydrochloric acid in the same non-secreting cat stomach.

TABLE III. Changes in concentration and volume on 30 min instillations of 170 mM hydrochloric acid into the cat stomach at secretory arrest. A 30 min instillation of 170 mM acetic acid was given between the first and second instillation of hydrochloric acid. The table gives the mean values of 5 expts., ± S.E.

Installation no.	ΔH mM	ΔCl mM	ΔNa mM	ΔK mM	ΔV ml
1	− 71 4	− 24 2	+ 46 ± 4	+ 5 ± 1	+ 0.4 ± 0.1
2.	− 114 ± 6	− 44 ± 1	+ 70 ± 4	+ 5 ± 1	+ 0.4 ± 0.4
3	− 98 ± 2	− 35 1	+ 65 ± 3	+ 4 ± 1	+ 0.5 ± 0.2
4	− 81 ± 4	− 29 1	59 ± 5	+ 4 ± 1	+ 0.4 ± 0.3

when the permeability had returned to the normal level. A new increase in permeability was observed.

Sodium acetate had a considerably smaller effect on the ionic permeability of the gastric mucosa.

When hydrochloric acid is instilled in a non-secreting stomach, the hydrogen ion concentration decreases according to the equation

$$H \quad H \quad \frac{-kt}{p}$$

TABLE IV. The permeabili y coefficients (ml/min) for hydrogen ions (k) and sodium ions ( ) on instillation of 170 mM hydrochloric cid before and after instillation of 170 mM acetic acid and of sodium acetate solution.

HCl 1 k		Interspaced instillation	HCl 2 k	S	HCl 3 k		HCl 4 k	
0 13	0.10		0.22	0.16	0 19	0 15	0.11	0 12
—	—		0.23	0.13	0.17	0 11	0.15	0.12
0.09	0.06		0.24	0 15	0 16	0.15	0 16	0.10
0 09	0 0?	Acetic acid	0.21	0 11	0.16	0 11	0 10	0 06
0.13	0.10		0.22	0.14	0.19	0 13	0 15	0.13
0 12	0.08		0 18	0.13	0.16	0 12	0 10	0.10
0 08	0.08		0.20	0.13	0.15	0 11	0 13	0 11
0.11	0 13		0.22	0.16	0.16	0 14	0.11	0 14
0 11	0.09	Mean	0.22	0.14	0 17	0 13	0.13	0.11
0.11	0.09	Sodium	0 13	0 09	0.13	0 09	0 13	0 09
0.10	0 08	acetat	0.15	0.09	0 10	0.07	0.10	0 0?
0 08	0 10		0 12	0 11	0 09	0.10	0 10	0.09
0.10	0 09	Mean	0 13	0 10	0.11	0.09	0.11	0.08

here H is the observed ion concentration (mM) at time H_0 the original hydrogen ion concentration, k the permeability coefficient for hydrogen ions (ml/min) and p the olume (ml)

The sodium concentration increases according to the equation

$$Na = S_e \left(1 - \frac{st}{p}\right)$$

where N is the actual sodium oncentration at time t, S_e the equilibrium concentration and the permeability coefficient for sodium ions. The alue for S_e is given as 140—150 mM (Terrell 1947 Öbrink 1948)

In the present calculations the alue 145 mM was used. The alues for k and from instillations of hydrochloric acid before and after instillation of cetic acid or sodium acetate are given in Table IV and Fig. 8 An nstillation of acetic acid increased the permeability coefficient for hydrogen ions by an verage of 100   The corresponding increase in the permeability coefficient for sodium ions was 55 + No significant difference was found between the permeability coefficients obtained n nstillation of hydrochloric acid 60—90 min after the instillation of cetic cid nd those obtained at the initial hydrochloric acid instillation The effect of the acetic acid on the ionic permeability of the gastric mucosa was thus reversible and did not persist for longer than about 90 min

Cont ol periments

In order to ascertain the extent to which residual sol tion f om the immediatel preceding instillation influenced the result samples wer taken min fter nstilla tion of a new solution. The changes n concent tio ttributable to 2 min diffusion were calculated and subtrated from those obtained n the n l ies It was found that

the hydrogen ion concentration decreased and the sodium ion concentration increased by only a few mM as a result of dilution. The effect of dilution was thus negligible.

In order to exclude the possibility that the permeability altered spontaneously during the experimental period, tests were carried out with five consecutive 30 min instillations of 170 mM hydrochloric acid. The result of such a test is shown in Fig. 9. The changes in concentration and the permeability coefficients were the same in all 5 instillations (range for the permeability coefficient ± 0.01 ml/min).

Microscopic examination

Three cats were anesthetized and operated on in the usual way. At the operation a specimen from the corpus of the stomach was taken for histological examination, after which 170 mM acetic acid was instilled into the stomach. 30 min later a further specimen was taken from the stomach wall. The specimens were immediately immersed in 10 % formalin. Hematoxylin and eosin stains were used. No difference was observed between sections from the two specimens.

Discussion

Migration of acetic acid and sodium acetat

Concerning the changes in ion concentration, the results obtained in model experiments with 170 mM hydrochloric acid were essentially the same as those obtained in the animal experiments, indicating that the mechanism of migration of hydrochloric acid was the same in both cases. This was first shown by Teorell (1947).

On instillation of acetic acid into non-secreting stomachs and in model experiments, there was an outward diffusion of acetic acid and an inward diffusion of sodium, potassium and chloride ions. In the model experiments the disappearance rate of acetic acid was lower than that of hydrochloric acid. This was contrary to what was found in the animal experiments in which the disappearance rate of acetic acid was the highest. These results indicate different mechanism of migration of the acid through the gastric mucosa and the cellophane membrane. It is in accordance with the theory that lipid soluble undissociated acids can pass through the lipid regions of the cell membrane. In the animal experiments the decrease in hydrogen ion concentration was significantly greater than the decrease in acetate concentration. The combined sodium and potassium ion concentration increased significantly more rapidly than the chloride ion concentration. This suggests that apart from molecular disappearance of acetic acid there was an exchange between hydrogen ions and sodium-potassium ions. The presence of secretion tended to mask the magnitude of this ion exchange. Evidence for this is given in Fig. 4 and Table I. After instillation of acetic acid into cat stomach, the volume of the intragastric solution decreased, which was probably due to the hypoosmolarity of the acid.

On instillation of sodium acetate into the stomach and in model experiments, an acetate-chloride exchange occurred. In the model experiments where the sodium concentrations in both compartments were initially the same the sodium concen-

tration increased in the inner solution (Fig 6 and Table II) This was probably a diffusion effect, since the mobility of the chloride ions is greater than that of the acetate ion (7.9×10^{4} and 4.3×10 cm/sec, respecti ely in infinite dil tion at 25 C) In instillations into the cat stomach the situation was more complicated due to the existence of a concentration difference between inner and outer solution" (170 mM—S i.e. about 145 mM) Experimentally the sodium concentration de creased somewhat (Fig. 5) which can be interpreted as the resultant of simultaneous differences in ionic mobilities and in concentration.

Effects of acetic acid and sodium acetate on the gastric transmucosal ion exchange

An important factor in the regulation of the acidity in the stomachs is the exchange diffusion between sodium and hydrogen ions (Teorell 1947 Öbrink 1948) On instillation of hydrochloric acid *into a cat stomach* the difference between the de crease in hydrogen ion concentration and the decrease in chloride ion concentra tion was approximatively equal to the increase n sodium and potassium concentration both before and after instillation of acetic acid. The net result of the effect of acetic acid on the mucosa may be interpreted thus as an increased diffusion ex change between hydrogen and sodium ions.

On the basis of the theory that lipophilic undissociated cids pass through the lipid regions of the cell membranes, it has been suggested that a varying degree of dissociation takes place intracellularly (dependent on the pK_a value of the acid) This would result in n intracellular liberation of hydrogen ions and, assuming that the efflux/metabolism is low an anionic accumulation Accumulation of ions in the cells would give rise to a transient intracellular pH reduction, intracellular hyper tonicity and swelling of the mucosa. W suggest that these changes might be the causes of the permeability increasing effect of acetic acid. It agrees well with the fact that the mucosal permeability for ions returned to normal in a relatively short time. Pathways for migration of sodium ions into the gastric l men are not known but evidence has been presented that the disappearance of hydrogen ions takes place in an extracellular route (Öbrink nd Waller 1965) It is possible that swelling of the mucosa increases the extracellular space. An increased blood flow in the mucosa due to transient local acidosis or histamine liberation (cf Johnson 1966, Johnson and Overholt 1967) may also be of importance of the rate of the exchange diffusion. The reversibility of the effect of acetic acid on the ionic permeability of the gastric mucosa would seem to mean that no f rther hydrogen ions enter the cells when th acetic acid instillation is discontinued.

It has been reported by Grant (1945) that acetic cid produces local erosions of the surface epithelium of the m sa Such erosions would diminish th diffusion distance and thereby increase th permeability It seems improbable however that such epithelial damage could be reversed during the 90 min period in which th stomach was in contact with 60—170 mM hydrochloric acid after exposure to acetic acid. No epithelial changes were observed on m cosa, which for 30 min had been in contact w th acetic acid.

It has been suggested that acetic acid stimulates the stomach to secretion of neutralizing mucus (Babkin 1950). Evidence has been presented that acetic acid instillation gives rise to an increased flow of serum proteins and blood into the gastric lumen (Davenport 1966). As no volume increase as an effect of acetic acid instillation was obtained (see Table III), it is considered less probable that an inflow of neutralizing fluids could account for the increased disappearance rate of hydrogen ions.

Compared with acetic acid, sodium acetate had a small permeability increasing effect on the gastric mucosa. This could have been due to that the stomachs were not in a state of absolute secretory arrest (see Fig. 4), which would have meant the formation of a small amount of acetic acid from sodium acetate and hydrochloric acid. Sodium acetate per se probably had no effect on the mucosal permeability.

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We were introduced to the problem by professor K. J. Obrink. We are very grateful to Mrs Lena Westlund for technical assistance.

References

Babkin, B. P., *Secretory mechanism of the digestive glands.* 2nd ed. New York 1950.
Babkin, B. P., C. O. Hebb and L. Krueger, Changes in the secretory activity of the gastric glands resulting from the application of acetic acid solutions to the gastric mucosa. *Quart. J. exp. Physiol.* 1941. *31.* 63—78.
Davenport, H. W., Gastric mucosal injury by fatty and acetylsalicylic acids. *Gastroenterology* 1964. *46.* 245—253.
Davenport, H. W., Fluid produced by the gastric mucosa during damage by acetic and salicylic acid. *Gastroenterology* 1966. *50.* 487—499.
Flemström, G., B. Frenning and K. J. Obrink, The disappearance of acetic acid and acetate from the cat stomach and its influence on the permeability for hydrochloric acid. *Acta physiol. scand.* 1964. *62.* 422—428.
Grant, R., Conditions under which the epithelial cells of the gastric mucosa are shed and disintegrate. *Canad. med. Ass. J.* 1944. *51.* 377—378.
Grant, R., Rate of replacement of the surface epithelial cells of the gastric mucosa. *Anat. Rec.* 1945. *91.* 175—185.
Johnson, L. R., Histamin liberation by gastric mucosa of pylorus-ligated rats damaged by acetic or salicylic acids. *Proc. Soc. exp. Biol.* (N.Y.) 1966. *121.* 384—386.
Johnson, L. R. and B. F. Overholt, Release of histamine into gastric venous blood following injury by acetic or salicylic acids. *Gastroenterology* 1967. *52.* 505—509.
Kalbhen, F. and J. Rezvani, Eine Schnellmethode zur Bestimmung von Tritium Radiokohlenstoff und Radioschwefel in beliebigem organischen Probenmaterial mittels der Flüssigkeits-Scintillations-Zählers. *Helv. chim. Acta* 1961. *44.* 1956—1966.
Martin, B. K., Accumulation of drug anions in gastric mucosal cells. *Nature* (Lond.) 1963. *198.* 896—897.
Obrink, K. J., Studies on the kinetics of the parietal secretion of the stomach. *Acta physiol. scand.* 1948. *15.* Suppl. 51.
Obrink, K. J. and M. Waller, The transmucosal migration of water and hydrogen ions in the stomach. *Acta physiol. scand.* 1965. *63.* 175—185.
Schanker, L. S., P. A. Shore, B. B. Brodie and C. A. M. Hogben, Absorption of drugs from the stomach. I. The rat. *J. Pharmacol. exp. Ther.* 1957. *120.* 528—539.
Teorell, T., Studies on the diffusion effect upon ionic distribution. I. Some theoretical considerations. *Proc. nat. Acad. Sci.* Wash. 1935. *21.* 152—161.
Teorell, T., Studies on the diffusion effect upon ionic distribution. II. Experiments on ionic accumulation. *J. gen. Physiol.* 1937. *21.* 107—122.
Teorell, T., On the stomach permeability for acids and some other substances. *J. gen. Physiol.* 1939. *23.* 263—274.
Teorell, T., Electrolyte diffusion in relation to the acidity regulation of the gastric juice. *Gastroenterology* 1947. *9.* 425—443.

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Diamine Oxidase in Blood Plasma in Some Vertebrates and Anodonta cygnea before and after Injection of Heparin

By

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Abstract

HANSSON, R. and H. THYSELL. *Diamine oxidase in blood plasma in some vertebrates and Anodonta cygnea before and after injection of heparin.* Acta physiol. scand. 1968. 74. 533—542.

After intravenous injection of heparin (in amounts related to the body weight) the blood plasma diamine oxidase (DAO-) activity increased transiently in all vertebrates studied (cod, frog, fowl, mouse, white rat, golden hamster, guinea-pig, rabbit, cat, dog, goat, sheep and cow). The DAO-response to heparin varied strongly between the species. Often with a biphasic ascent, it showed a maximum (within a few minutes to about one hour) and then decreased, often in a "monoexponential" fashion, in the course of the next few hours. In anodonta no significant DAO-activity was found.—The biological significance of the heparin effect upon DAO is discussed with special reference to the coexistence of heparin and histamine in the mast cells.

The coexistence of various pharmacodynamically potent substances, especially histamine and heparin, in the mast cells and their simultaneous release in anaphylactic and other conditions, have stimulated to many interpretations (e. g. Riley 1962, 1963 and 1964, Selye 1965, Sagher and Even-Paz 1967 for references). Observation made in guinea-pigs (Bernauer *et al.* 1964) and independently thereof in human beings (Tryding 1965 a) indicate another relationship between heparin and histamine. In man, after i. v. injection of heparin, the blood plasma diamine oxidase[1] (DAO) activity was shown to rise transiently from normal low levels to very high ones (from about 0.015 to 1 or more enzyme units per liter plasma). This latter finding prompted us to see if the influence of parenterally administered heparin on the blood plasma DAO-activity was a generally occurring phenomenon among the vertebrates. As there are considerable species differences in the histamine metabolism—formation, organ sensitivity and catabolism—(Kahlson and Rosengren 1968 for discussion and

[1] No attempt has been made to distinguish between diamine oxidase (DAO) and histaminase which are used synonymously in the text according to the Commission of Enzymes of the International Union of Biochemistry (for No. 1.4.3.6 diamine: oxygen oxidoreductase (deaminati

TABLE I Material and methods

Species	Body weight (BW)	Number of animals	experi-ments
Anodonta cygnea (pondmussel)	70—80 g	6	6
Gadus morrhua (cod)	430—700 g	4	4
Rana temporaria (frog)	20—25 g	2	2
Gallus domesticus (domestic fowl)			
Gallus domesticus (hen)	2.5—2.9 kg	2	2
Gallus domesticus (cock)	2.4—3.0 kg	2	2
Mus musculus (mouse)	35—65 g	6	6
Mus Norvegicus (white rat)	230—370 g	4	4
Mesocricetus auratus (golden hamster)	85 g	1	1
Cavia cobaya (guinea-pig)	660—790 g	4	4
Oryctolagus cuniculus (rabbit)	2.0—2.2 kg	6	6
Felis catus domestic (cat)	1.5—1.9 kg	2	4
Canis familiaris (dog)	12—17 kg	2	5
Bos taurus (cow)	400 kg	1	1
Capra hircus (goat)	50 kg	1	1
Ovis aries (ram)	60 kg	1	1

references) as well as in the amount and distribution of DAO (Zeller 1949 and 1963) the quantitative aspects and time-relations were also considered. No acceptable evidence exists for the occurrence of mast cells in invertebrates (*e.g.* Selye 1965). To get an indication of whether a DAO-releasing effect of heparin nevertheless occurs among them, the *Anodonta cygnea* (pond mussel) was also studied. The vertebrate series included cod, frog, fowl, mouse, rat, golden hamster, guinea pig, rabbit, cat, dog, sheep, goat and cow.

Material and methods

Material and methods. All the experiments were performed on healthy animals. Xylocain® 1 % 1—5 ml, was used for local anesthesia during the cannulation. For the sake of brevity the experimental material is described in tabular form (Table I). Blood (100 µl or more) was sampled by the short intraarterial polyethylene cannula (with due attention to the dead space) into heparinized centrifuge tubes (Sarstedt type) or by puncture with cannula and syringe. The blood samples were within 30 min after the collection, centrifuged and plasma separated and stored at −20 °C until analysis. The activity of the diamine oxidase (DAO) was determined according to the method of Okuyama and Kobayashi in modification for ultra-micro samples by Tryding (1965 b).

Throughout the experimental series was used Heparin ®—(Vitrum) 5000 I.U./ml of water preserved with tricresol 0.2 %, in some cases diluted with sterile saline 0.9 %. (Control injection of tricresol in "equivalent" amounts into guinea-pigs did not increase the activity of DAO in the blood plasma.) Further details on the experimental procedures may be obtained from the authors, after request.

Anesthesia	Heparin dose, U/g BW	Site of heparin injection	Site of blood sampling
none	3.0	vascular sac	vascular sac, puncture
none	0.2 1.6	bulbus arteriosus	rteriosus bulb, cannula
local	1.6	left aorta	left aorta, catheter
local	1 7; 2.0	alar vein	alar vein, puncture
local	1 6	cartoid artery (c.a.)	c.a., cath.
ether	1.6	c.a	c.a., cath.
none		periton., subcut.	heart, punct.
ether	1.6	c.a.	c.a., cath.
ether	3.2	c.a.	c.a., cath.
local	0.2 1 6	c.a.	c.a., cath.
local	0.2 0.4 1.6	jugular or femoral vein[4]	c.a. fem a., cath.
ether	0.2 0.4 1.6	jugular or femoral vein	c.a. fem. a., cath
none	0.2 0.4	cephalic vein[5]	ceph. ein, cath.
none	0.2	jugular vei	jug vein, cath.
local	0.4	jugular vein	jug vein, cath.
local	0 2	jugular vein	jug vein, cath.

Notes 1 Two mice. 2. Three mice. 3. One mouse. 4 Heparin was given in decreasing dose (1 6, 0.4 and 0.2 U/g) to one cat, in increasing dose to he other 24 hrs between each experiment 5. One dog was given 0.2 and 0.4 U/g t 24 hrs interval, the other only one dose of 0.2 U/g. 6. The three heparin doses were given according t "latin square arrangemen The interval between the injections was 24 hrs.

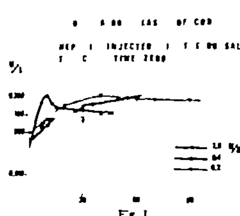
Results

Anodon No significant change of the very low DAO-activity in the "blood could be registered up to one hour after the heparin injection. Neither did the homogenate (with diisopropylphosphofluoridate, DFP to a concentration of 10^{-3} M) show any significant DAO-activity

Cod (Fig. 1) The reference samples showed a DAO-activity of 0 03—0.05 U/l The injection of heparin was followed within 10 min by n increase of DAO in the blood plasma. The highest ctiv ty was recorded at different intervals between 10 and 60 min after the injection. The increase was about 5-fold after 0.4 and 1 6 U heparin/g but only about 2 fold after 0.2 U/g body weight The DAO-activity of the blood plasma remained elevated, also after the smallest dose, during the observation period (90 min)

Frog The DAO-acti ity values were initially 0 04 and 0 08 U/l, and fter the injection of heparin they rose to almost 5 U/l, after which they tended to decrease

Fowl (Fig 2) The basal DAO-level in the blood plasma in hen as well as cock was 0.03—0.07 U/l. E en in the first sample collected after the injection of heparin



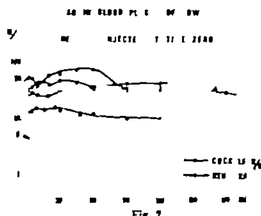


Fig 1 Fig 2

Fig 1 *Gadus mor hua* (od) Abscissa Time (in minutes) after injection of Heparin® (V trum) Ordinat Diamine oxidase (DAO) activity in U (µmoles putrescine consumed per min) per liter (L) blood plasma.

Fig 2 *Gall domestica* (Fowl)

(75 sec to 5 min) the DAO-activity had risen to almost maximum level at 500 to 1000 times the initial values in the hens and more than 1000 times in the cocks, that had received a somewhat larger dose of heparin. During the rest of the observation period (about 180 min) the DAO-activity in the blood plasma was very high several hundred times the basal level.

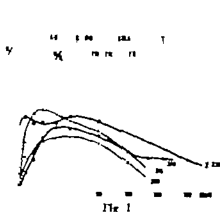
Mouse (Table II) The basal values ranged from 0.01 to 0.06 U/L I v injection of heparin, 1 6 U/g was followed by a more than hundredfold increase of the DAO (to 8.3 2.5 and 4.5 U/l respectively) Neither i.p. nor s.c injection of the same relative doses of heparin produced more than a barely significant increase.

White Rat (Fig 3 Before the injection of heparin (1 6 U/g) the DAO-activity varied between 0 01 and 0 04 U/l plasma After 5 min it had increased 6- and 21

Table II DAO-activity in Mouse before and after heparin, 1 6 U Heparin®/g bodyweight

Rout	Intraperit			Sub-cut	Intraven.			
Mouse no	1	2	3	4	5	6	7	8
Before heparin	017	017	0 14	.024	.012	064	—	—
30 min after heparin	019	030	024	018	—	—	8.30	—
50 min after heparin		—	—	—	—	—	—	2 5
60 min after heparin	023	041	033	018	—	—	—	
70 min after heparin	—		—	063	—	—	—	4 5
90 min after heparin	—	026	0.4	—	—	—	—	
100 min after heparin	.044	—	—	—	—	—	—	

Killed during anaesthesia


Fig 1

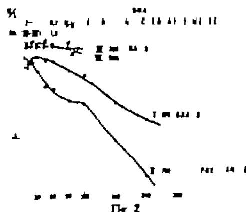

Fig 2

Fig. 3. *Mus norv. gicus* (white rat)
Fig 4. *Cavi obey* (Guinea-pig)

fold in ? animals, and after 10 min the average increase was at least 20 times the initial mean activity In one of the animals the maximum was distinctly biphasic. The decrease in activity had a "half time" of almost one hour Two hours after the injec tion of heparin the DAO-activity in all the animals was still 10—20 times higher than the initial value.

Golden hamster The initial ctivity of DAO was about 0.03 U/l nd rose to 3.5 U/l within 10 min after heparin. 30 min later it fell to about half this level and then increased to reach a new peak, of roughly the same level as th first During the entire experimental period (120 min) the enzyme level was more than 100-fold the initial value

G inea-pig (F'g 4) The DAO-activity of the blood plasma varied initially be tween 0.08—0 50 U/l with the lowest alue n pregnant animal (no. II) After in- jection of heparin the activity rose rapidly n one case 100-fold within 2 m n. In the animals that had received 0 2 U heparin/g it reached a maximum of about 18 U DAO/l, and then declined with half time of 30—35 min in the nimals given 1.6 U heparin/g the peak enzyme ctivity exceeded 30 U DAO/l and stayed t a plateau during the 90 min of sample collection.

Rabbit Within 10 min after the administration of heparin (0.4 U/g) the DAO- activity of the plasma rose from about 0 015 to about 0 055 U/l. After the first steep rise a shelf" level was rea hed and then a new increase (t bout 0.1 U/l) occ rred to a maximum, roughly 60 min after the injection. When a higher dose of heparin was given (1 6 U/g) the peak blood plasma DAO- tiv ty was on the average bout 5 U/l, i.e. about 40 times the maximal acti ity noted after the smaller dose of heparin. The maximum activity was also somewhat delayed nd finally the declining phase was less teep The "half time" was about 75 min against about 40 min fter the smaller dose of heparin. (H nson and Thysell to be published)

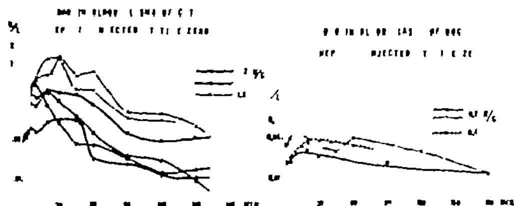




Fig. 5 Fig 6

Fig. 5 *Feli atus (domest c)* Cat. Response to different doses of Hepari ●
Fig. 6. *Ca is familiaris* (Dog mongrel

Cat (Fig 5) The DAO-activity in all the animals was initially between 0 01 and 0.0? U/l blood plasma. After heparin (1 6 U/g) it rose to about 5—30 times the initial value within 2—3 min. After 0 2 and 0.4 U heparin/g respectively the individual curves overlapped considerably but the responses were smaller than after the larger dose. The DAO-response was biphasic in 3 of the 6 assays, in the fourth there was an initial shelf and only in ? of the experiments an even rise to the maxi- value occurred. The return to normal level of the DAO-activity showed a half time of about 30 min in all the groups.

Dog (Fig 6) When 0.2 U heparin/g was given, the plasma DAO-activity rose to a maximum 3 to 4 times the initial value, which was 0 01 and 0.0? U/l. An increase of the dose to 0 4 U/g was not followed by a larger response

Cow (Fig 7) The DAO-activity of the plasma rose from 0.07 to 16 U per liter *i.e.* about 200 times in the first 2 min after the injection of 0.2 U heparin/g A maxi

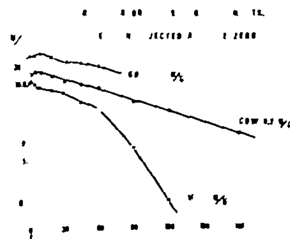


Fig 7 *Bo (taurs)* Cow Calf *hir us* Goat. *O is aries*. Ram.

mum was reached already within 5 min of the injection of heparin, after which the DAO-activity fell apparently linearly in the logarithmic diagram with a half time of barely 50 min.

Goat (Fig. 7). The DAO-activity of the blood plasma showed a basal value of 0.02 U/l. Within 2 min after the i.v. injection of heparin (0.4 U/g) it rose more than 1000-fold (to a little more than 33 U/l), reached a maximum within 20 min and remained high during the observation time.

Ram (Fig. 7). Before the injection of heparin (0.2 U/g) the plasma level of DAO was about 0.004 U/l but within min of the injection it reached a maximum level of nearly 12 U/l, i.e. more than some thousandfold the original level. The descending phase poorly defined a half time or some tens of minutes.

Discussion

Discussion. The only invertebrate in this study showed no significant amounts of diamine oxidase (DAO) in the "blood" before or after heparin injection and no such activity in the soft tissue homogenate. These findings agree with reports that no DAO has been found in Sepia officinalis (Blaschko 1941, 1963) or Eledone cirrhosa (Boadle 1967)—whereas, in the former species, amine oxidase and, in the latter species, a histamine-destroying enzyme without characteristics of DAO was present.

Throughout the vertebrate series in our investigation the heparin injection was followed by a transient rise of the blood plasma DAO. This reactive pattern, thus, seems to be a general finding in the vertebrate kingdom. This is in contrast to earlier reports (Schmutzler 1966), where the guinea pig was stated as the only species with this reactivity. Some of our findings have later been confirmed (Schmutzler *et al.* 1966).

However, it is also obvious, that there are considerable species-differences in the blood plasma increase of DAO after intravenous injection of heparin. The reaction was poor in the cod but also in the dog. In contrast to the latter, the other carnivorous animal (the cat) responded more strongly to the same doses of heparin. A marked rise of the blood plasma DAO activity was seen not only in the guinea-pig but also in the fowl group and, above all, among the ruminants. We have not been able to obtain a purposeful correlation between the species-bound variation in the DAO response to heparin on one side and on the other phenomena such as sensitivity to histamine (for review see Narjano 1966), tendency to anaphylactic reaction, the catabolic routes of histamine (Schayer 1966), sex, body-temperature or reproduction. The enzyme determination system does not exclude a species-bound variation in substrate-specificity. Some of our results have, however, been confirmed with histamine as substrate (*Schmutzler et al. 1966*). *Several factors, possibly in combination,* may contribute to these differences. The effect of heparin-binding to plasma proteins, the distribution of injected heparin among different organs, the amounts of mobilizable DAO in these (Biffom 1966, for references), the transport of liberated DAO to the blood plasma and the rate of DAO-elimination from the

plasma may thus vary between the species. Present knowledge of these factors does not warrant a discussion on a quantitative basis.

Schmutzler (1966) has clearly demonstrated that, in the guinea pig the increase of DAO ("histaminase") in blood plasma after administration of heparin is predominantly due to a release of the enzyme from the liver which is almost emptied of this enzyme after appropriate doses. The rapid increase of the DAO in blood plasma after i.v. heparin injection in our series suggests that part of the enzyme releasing structures are in close connection with the blood stream, from which they can be influenced by the heparin and into which some of the released DAO-activity can enter directly.

The further parts of the DAO-curves are more difficult to interpret. Several individual experimental animals develop a biphasic ascent of DAO activity like that often encountered in man. In other animals—and apparently independent of body size (see guinea pigs and ruminants)—the first increase is the only discernible one. It has been shown that significant amounts of the DAO are transported by the lymph in man (Hansson *et al.* 1966 data to be published). In three human subjects the thoracic duct lymph was diverted from the systemic circulation, which resulted in a monophasic and rather low blood plasma curve of DAO-activity after heparin injection (Dahlbäck *et al.* 1968). The time of maximal DAO-activity in the thoracic duct lymph corresponded well to the time of the peak activity in human subjects with intact lymph flow. Analogous findings have been made in rabbit, rat and guinea pig (Hansson and Thysell, data to be published). The biphasic ascent is compatible with a double contribution of DAO to the blood plasma, the first being due to a direct secretion, the second might depend on delayed transport of more or less large amounts of the enzyme. If this delay is shortened (by increased lymph-flow or shunts) or if the first phase of the curve is large as compared to the second one the latter may not be discernible. The rate of descent of the curve expresses the difference between the elimination from and the tapering DAO-contribution to the blood plasma. In spite of a rather monophasic appearance it does not describe only the eliminatory rate of DAO from the blood plasma. The eliminatory routes and mechanisms are, however, not known. That no excretion of the enzyme occurs by the urine has been reported (Kahlson 1956) and has been confirmed by us in a few pilot measurements on heparin-stimulated rabbits. There is a measurable increase in the DAO-activity of rabbit bile but not sufficient to explain the disappearance rate (To be published).

Other factors influencing the increase of blood plasma-DAO are the route of heparin injection (as shown in mouse —a phenomenon also seen in man— and the heparin dose (Schmutzler 1966, Hansson *et al.* 1966).

The biologic importance of the heparin-induced DAO-release is not clear. There is, however, a possibility that the heparin liberated after the mast cell-disintegration may release DAO which will hunt the histamine in an effort to protect remote histamine-sensitive structures from excess histamine. An increase of a "histamine-destroying factor" has been demonstrated during anaphylaxis in the rabbit (Rose

and Leger 1952) in the white rat (Code *et al.* 1961) and—as soon as 15 to 30 sec after the challenge dose—in the guinea pig (Logan 1961). Also in man, after anaphylactic shock, a high activity of plasma histaminase has been reported (Guidotti, L., quoted by Bufforni 1966).

In the guinea pig a metachromatic substance is released into the systemic circulation during anaphylaxis. This substance has a high histaminase-releasing activity which can be neutralized by protamine. When injected into the sensitized animal before or immediately after the challenge dose protamine will prevent the DAO-increase in blood during anaphylaxis (Schmutzler *et al.* 1967). In the guinea pig this heparin (-and?) substance seems to be essential for the DAO-release in anaphylactic shock. It is possible though not proven, that in species where heparin has not, as yet been identified in blood plasma during anaphylaxis (rabbit, rat, man) there is a local effect of released heparin on DAO-binding structures. On the other hand the presence of anaphylaxis-released, large amounts of heparin in the blood stream does not seem to warrant a marked rise of blood plasma DAO as—in dog—the former phenomenon occurs but (after heparin injection) a poor response in the blood plasma-DAO is noted.

Histamine is known as a lymphagogue, and it has been shown that during anaphylactic shock the central lymph flow in rat is increased several times (Logan 1960). This effect might promote a rapid infusion of lymph-borne DAO into the blood stream.

The protective effect of the mobilization of DAO in guinea-pigs during anaphylactic anaphylactoid and histamine shock has recently been elucidated by Giertz *et al.* (1967). Heparin also exerts a protective effect on rabbits during anaphylaxis when injected 30 min before the challenge dose (Dhar *et al.* 1967).

Owing to the effect of heparin, the role played by the mast cell as an emergency kit should be extended to include one further function. Disintegration of mast cells is accompanied by a release of histamine, which increases the permeability of the regional capillaries, and of heparin, which will mobilise DAO. Histamine being a lymphagogue, accelerates the transport of DAO to the blood stream, the blood capillary permeability to DAO-molecules may perhaps be affected. This transport system appears to secure a rapid increase of DAO in the plasma and maintenance of the high concentration in the lymph and in the blood.

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The expert performance of the DAO-analyses by mrs Gulli Lindfors is deeply appreciated.

References

BECHAUER, W., H. GIERTZ, F. HAHN, W. SCHMUTZLER, O. SEIDEL and B. SCHULTZ. Zum Mechanismus der Histaminfreisetzung im anaphylaktischen Schock. *Naturwissenschaften* 1964 *51* 412—413.

BLASCHKO, H., Amine oxidase in Sepia officinalis. *J Physiol.* (Lond.) 1941 *99* 364—369.

BLASCHKO, H., Amine oxidase. *The Enzyme*. Eds. J. B. Sumner and K. Myrbäck. Academic Press, New York, 1963. Vol. *8* 337—351.

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From the Department of Physiology University of Göteborg, Sweden

The Effects of Hypercapnia and Hypoxia on the Distribution of Capillary Blood Flow in the Denervated Intestinal Vascular Bed

By

JOAR SVANVIK, JÖRAN TYLLSTRÖM and INGEMAR WALLENTIN

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Abstract

SVANVIK, J J TYLLSTRÖM and I WALLENTIN *The effects of hypercapnia and hypoxia on the distribution of capillary blood flow in the denervated intestinal vascular bed* Acta physiol. scand. 1968 *74* 543—551

Experiments are described which demonstrate that both hypercapnia and hypoxia exert potent relaxing influence on the resistance vessels of the denervated intestine The effects on the capillary fluid exchange capacity, as indicated by the capillary filtration coefficient (CFC) are, however more complex. During local hypoxia CFC increases considerably probably reflecting direct relaxing influence on the precapillary sphincter section. I contrast, during hypercapnia CFC decreases in spite of a augmented blood flow This is assumed mainly to be due to an increased myogenic activity in the same sphincter section, elicited locally by raised transmural pressure and blood flow Functional implications of the results are briefly discussed. They suggest that neither high P_{CO_2} accompanied by low pH, nor low P are of major importance for a functional intestinal hyperemia during digestion.

The effects of hypercapnia and hypoxia on th intestinal vascular bed have been explored in several respects. It seems well established that, with regard to the resistance vessels, there is a competition between a local dilating infl ence on the one side and a nervously mediated vasoconstriction on the other (Grim 1963) Studies on the vascular effects of changes in P_{CO_2} have shown that although a pe ripheral dilator effect seems to be potent already t eucapnia (M c Dowall 1930 Sidky and Bean 1951) it is normally opposed by an external vasoconstrictor influence, and at increasing degrees of hypercapnia the intestinal flow resistance will for this reason gradually increase (Brickner *et al* 1956, Epstein *et al* 1961) At marked hypercapnia however the local dilating action of CO_2 will tak over and the intestinal flow resistance then begins to fall again (Brickner *et al.* 1956) One reason for this may be the relatively weak vasoconstrictor fibre infl ence that has been observed in this vascular bed (Bernthal and Schwind 1945 Sidky and Bean 1951 Bea and Sidky 1957 for further ref see W llentin 1967)

BOADLE, M. Observations on a histaminase of invertebrate origin, the renal appendages of Eledone cirrhosa. *J. Physiol.* (Lond.) *1967 192* 33—36.

BUFFONI, F. Histaminase and related amine oxidases. *Pharmacol. R.* 1966. *18* 1163—1199.

CODE, C., D. COOY, M. HURN, J. KENNEDY and M. STRICKLAND. The simultaneous release of histamine and a histamine-destroying factor during anaphylaxis in rats. *J. Physiol.* (Lond.) 1961 *156* 207— 16.

DAHLBÄCK, O., R. HANSSON, G. TIBBLING and N. TRYDING, The effect of heparin on diamine oxidase and lipoprotein lipase in human lymph and blood plasma. *S and J lin. Lab. Invest 1968 21* 17—23.

DHAR, H. L., B. MUKHERJEE and R. K. SANYAL, The effect of heparin on the heart in anaph laxis. *Amer. H art J* 1967 *74* 489—493.

GIERTZ, H., F. HAHN, O. SEIBERT and W. SCHMUTZLER, Über die Wirkung on Heparin und Aminoguanidin auf den anaphylaktischen, Anaphylatoxin- und Histamin-Schock des Meerschweinchens. *Nau y -S hmied berg A h. exp Path. Pharmak* 1967 *256* 26—39.

HANSSON, R., C. G. HOLMBERG, G. TIBBLING, N. TRYDING, H. WESTLING and H. WETTERQVIST. Heparin-induced diamine oxidase increase in human blood plasma. *Act med sc d* 1966. *180* 533—536.

KAHLSON, G. The significance of histamine in the body. *Cib Fou dation Symposium Histam e* Eds. G. E. W. Wolstenholme and C. M. O'Connor. London, J. & A. Churchill Ltd. 1956, pp. 248—257.

KAHLSON, G. and ROSENGREN, E. New pproaches to the physiology of histamine. *Physiol Rev* 1968. *48* 155—196.

LOGAN, G. *Histamine in intestinal lymph of whit rat during anaphylactic shock. P oc Soc xp Biol.* (N.Y.) 1960 *104* 532—536.

LOGAN, G. Release of histamine-destroying factor during anaphylactic shock in guinea pigs. *Proc Soc p Biol* (N.Y.) 1961 *107* 466—469.

NAHJANO, P. Toxicity of Histamine. Lethal Doses. *Handbook of Experime tal Pharmacology* Eds. O. Eichler and Farah, A., Springer Verlag. Berlin, Heidelberg New York 1966. *XVIII/1* 179—197.

RILEY, J. F. Histamine and heparin in mast-c lls. Why both. *Lancet* 1962. *II* 40—41.

RILEY, J. F. Functional significance of histamine and heparin in tissue mast cells. Ann. N.Y. *A ad Sci* 1963 *103* 151—164.

RILEY, J. F. Mass cells and anaphylaxis. *Lan et* 1964 *I* 1155—1157.

...ON, B. and J. LAGER, Serum histaminase during rabbit anaphylaxis. *Proc Soc p Biol.* (N.Y.) 193 9 379—381.

SELYE, F. and Z. EVEN-PAZ *Mastocyt a d th mast* ll. Karger Basel/New York 1967.

SCHAYER, R. Catabolism of Histamine in i *H ndbook of E perimental Pharmacology* Ed. O. Eichler and Farah. A. Springer Verlag, Berlin, Heidelberg N.Y. 1966. XVIII/1 67—683.

SCHMUTZLER, W. *St die über die Histamin ase-F eisetz g beim Meerschweinchen im anaphylaktischen S hock d d k Hist m Liber ator* Dissertation Universitat Freiberg i.Br. 1966.

SCHMUTZLER, W., H. GIERTZ, F. HAHN and P. KRELL, Zur Frage der Heparin-Beteiligung bei der anaphylaktischen Histamine Freisetzung des Meerschweinchens. *Naunyn-Schmiedebergs Arch p Path Pharmak* 1967 257 59—60.

SCHMUTZLER, W., F. HAHN, O. GOLDSCHMIDT and G. SEIBERT, Untersuchungen über Substanzen mit histaminasefreisetzender Wirkung beim Meerschweinchen. *Naunyn-Schmiedebergs A h. p Path Pharmak* 1966 235 344—352.

SELYE, H. *Th mast Ils* Butterworths Washington. 1965.

TRYDING, N. (a) Heparin-induced diamine oxidase (DAO) activity. *S and J Cl Lab. Invest* 1963 *17* Suppl 86 196.

TRYDING, N. (b) Micromethod for determination of plasma diamine oxidase. *S d J b Lab I st* 1963 *1* Suppl 86 19.

ZELLER, A. Diamin-Oxydase. *Advances in Enzymology* Interscience Publ., New York, 1942 2. 93—112.

ZELLER, A. Diamine Oxidases. *The E zym* Eds. J. B. Sumner and K. Myrbäck. Academic Press, New York 1963. I *of* 2 313—335.

Hypercapnia was induced by ventilating the animal with 8—15 per cent CO_2 in O_2. The pH was measured with a glass electrode pH-meter (Radiometer type PHM 4C, Copenhagen) in small arterial samples (0.1—0.4 ml) taken from the femoral artery. In some experiments the carbon dioxide tension of the arterial blood (Pa_{CO_2}) was determined according to Astrup (1956). In most cases, however, the P_{CO} was continuously measured by diverting the arterial blood flow from one of the femoral arteries to a P_{CO}-electrode (Beckman B 160) placed in temperature controlled water bath, and back to the animal again. Comparison with the P_{CO}-values obtained by the Astrup technique showed a fairly good agreement.

Hypoxia was induced by gradually increasing the nitrogen tension of the inspiratory gas mixture until the intestinal blood flow increased to about the same magnitude as during the hypercapnia experiments. This procedure was accomplished in 5—10 min. It was not possible in these experiments to determine the P of the blood. Instead the P_O of the inspired gas was measured intermittently by means of paramagnetic oxygen tension analyzer (Beckman model D oxygen analyzer). Usually it was reduced to bout 50—60 mm Hg.

Results

Hypercapnia. Fig. 1, left panel, illustrated the typical effects of hypercapnia on arterial blood pressure, intestinal blood flow, intestinal volume and capillary filtration coefficients. By ventilating the animal with 12 % CO_2 the Pa_{CO_2} increased from a resting value of about 20 to approximately 80 mm Hg. Thereby the flow increased to about 250 % of control and then remained at this level throughout the period. Since the perfusion pressure was essentially unchanged this implies a decrease of the blood flow resistance down to about 40 % of its resting value. Despite this extensive flow increase, the intestinal volume was fairly constant. After a moderate initial increase, concomitant to the augmentation of the flow, the volume curve soon regained its previous slope.

Fig. 1, left panel, also illustrates the effects of hypercapnia on the capillary surface area, as estimated from the capillary filtration coefficient (CFC). From a control value of 0.086 ml/min × 100 g × mm Hg CFC decreased to about 0.075 during the period of blood flow increase.

The mean maximal blood flow increase as a result of hypercapnia was 76 (17 periods of hypercapnia), while the arterial blood pressure was not significantly altered. In spite of the dilatation of the intestinal resistance vessels, the precapillary sphincter appeared to constrict during the period of hypercapnia, since the capillary filtration coefficient decreased by an average of 19 %. This decrease was statistically significant ($p<0.05$) and it seemed to be more pronounced at moderate blood flow increases (up to 80 %) than at very extensive ones. Thus a very marked increase of blood flow was usually associated with a small CFC change (cf. Fig. 1 left panel).

During the elevation of the venous outflow pressure, induced when measurements of CFC were performed, small increases of blood flow resistance were usually seen. This reaction, described by Johnson (1959, 1965) as a "venous-arteriolar response", has been ascribed to an increase of myogenic tone of the precapillary resistance vessels, induced by the raised intravascular distending pressure. In the present experiments the sudden increase of venous outflow pressure by 10 cm water raised the blood flow resistance by about 10 % during both the control and hyper-

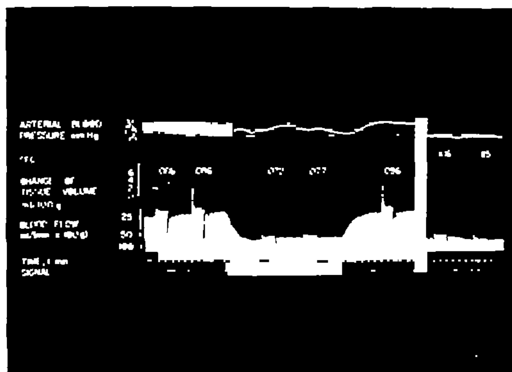


Fig. 1. Cat 3.5 kg. Pentobarbitone, gallamine iodide, artificial ventilation with pure oxygen. *Left panel.* During the period, marked by the signal, the cat breathes a gas mixture consisting of 12 % CO_2 in O_2, increasing arterial P_{CO_2} from about 20 to 80 mm Hg. Notice the large increase of the venous outflow concomitant with a moderate decrease of CFC. Intestinal tissue volume remains remarkably constant during this dilatation.
Right panel. Effect on CFC of intraarterial infusion of isopropyl-noradrenalin, producing the same extent of blood flow increase as produced by hypercapnia in left panel. Note how in this situation CFC increases in parallel with the blood flow increase.

capnia periods. This indicates that at least some arteriolar responsiveness to pressure changes was retained in spite of the dilating effect of the raised Pa_{CO_2}.

During control periods the pH values was in the order of 7.30—7.40 and during intense hypercapnia (60—70 mm Hg) it decreased to 6.90—7.10.

Fig. 1 right panel shows the effects of a close intra-arterial infusion of isopropyl-noradrenaline in the same experiment as that shown in left panel. During a blood flow increase comparable to that induced by hypercapnia CFC increased to about 0.106—0.115 ml/min × 100 g × mm Hg. Thus the CFC change was in the *same* direction as the blood flow in contrast to the situation during hypercapnia. This response to isopropyl-noradrenaline is in agreement with earlier results (Folkow *et al.* 1963) suggesting that this dilator drug relaxes the smooth muscles both in the resistance and sphincter sections.

Hypoxia. A typical effect of hypoxia on the intestinal vessels is illustrated in Fig. 2. The oxygen tension of the inspiratory gas ($P_{I_{O_2}}$) was decreased to about 5 mm Hg

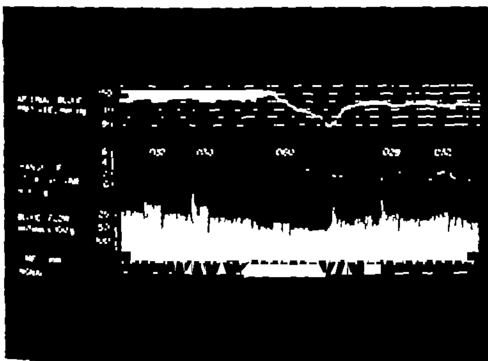


Fig. 2. Cat 3.2 kg. Pentobarbitone; gallamine iodide; artificial entilation with pure oxygen. During the period, marked by the signal, the cat breathes gas mixture consisting of O_2 in N_2 (partial O_2 pressure around 55 mm Hg; the CO_2-tension of the arterial blood re maining constant. Note the marked increase of CFC and the augmented blood flow despite gradually reduced arterial blood pressure, indicating continuously falling vascular resistance.

The blood flow showed a gradual increase to approximately 220 % of its original value and stabilized at that level. After a small initial rise, the arterial blood pressure declined at a largely constant intestinal blood flow revealing a continuous reduction of tone of the resistance essels. Simultaneoul CFC increased from 0.031 ml/min × 100 g × mmHg to 0.060 by about 100 % indicating a corresponding relaxation of precapillary ph ncters. The tone of the capacitance essels did not change appreciably n this experiment as judged by the f irly constant tissue volume during the period of low oxygen tension.

The a erage response to h poxia th present study was an increase of the intestinal blood flow w th 48 f th control lue (n = 9) Concomita tly there was proportional increase of CFC mount ng t 63 o as a mean. Although the intestinal volume changes were small in the experiment shown in Fig. 2, moderate increase of the in estinal vol me wa often seen d ring hypoxia, probably caused by a rise of the mean capillary pres re (see discussion). The pH of th rteri l blood did not change ppreciably during the rather brief periods of hyp xia.

The enous-arterial r response me tioned abov w much more inc nsi tent

during hypoxia than during hypercapnia and could not be seen in about half of the hypoxia experiments.

It should be stressed that all the effects of hypoxia mentioned here appeared to be far more varying than those of hypercapnia, presumably mainly due to a depression of the myocardium, which led to such a fall in arterial blood pressure that reliable measurements of the intestinal vascular adjustments could often not be made. As a rule it was necessary to lower the P_{IO_2} down to about 50—60 mm Hg to reduce intestinal blood flow resistance to the same extent as during the hypercapnia periods. The degree of the vasodilatation appeared to be affected by a variety of factors, such as rate of induction of hypoxia, the occurrence of previous periods, hematocrit, etc. Consequently the vascular changes to hypoxia showed a considerable variability.

Discussion

The present results indicate that the denervated intestinal vascular bed responds differently to hypercapnia and hypoxia. Both hypoxia and hypercapnia produce a marked relaxation of the resistance vessels, presumably by a direct effect on the vascular smooth muscle. However the venous-arteriolar response seems to be better preserved during hypercapnia than during hypoxia, suggesting that the myogenic reactivity of the resistance vessels to pressure changes is more compromised during oxygen lack. This would imply an impaired ability during hypoxia for such and postcapillary resistance adjustments that normally tend to keep the mean ary pressure constant despite changes of arterial or venous pressures.

The capillary filtration coefficient increases considerably under the influence of hypoxia, as well as under the influence of the vasodilator drug isopropyl noradrenaline but during hypercapnia it is reduced significantly. This tendency for CFC to decrease, in spite of an augmented blood flow during hypercapnia is most pronounced at moderately increased blood flow levels. At larger blood flow increases, induced by more intense hypercapnia, the CFC decrease is usually less pronounced.

This discrepancy in the responses of the precapillary sphincters at comparable degrees of intestinal blood flow augmentations during hypoxia and hypercapnia, respectively calls for some comments. Firstly an increased capillary permeability brought about by the graded hypoxia, might be responsible for the observed increase of CFC in this situation. Considering however the rather rapid development and the prompt reversal of the CFC responses during both hypoxia and hypercapnia this seems less probable (See Fig. 1 and 2). Further according to Landis and Pappenheimer (1963) far more extensive changes of P_{O_2} and of pH appear to be necessary to increase capillary permeability significantly. Secondly the tone of the precapillary sphincters may be affected to different degrees in the two situations. Hypoxia, as well as isopropyl noradrenaline might exert a more powerful relaxing influence on the precapillary sphincters than hypercapnia at a comparable

decrease of the total vascular resistance. This appears to be the most probable explanation for the marked increase during hypoxia, but it cannot explain the actual reduction of CFC observed during hypercapnia.

The reason for a contraction of the precapillary sphincters in connection with hypercapnia remains obscure, but there are two main possibilities. An increased wash-out of vasodilator metabolites caused by the augmented blood flow may enhance the tone of the sphincters so as to override a direct relaxing effect of the increased CO tension. Further the sphincter response may be due to a largely preserved myogenic reactivity during hypercapnia. It has been shown that the capillary filtration rate can decrease considerably when mean capillary pressure is raised, both in the limbs (Mellander Öberg and Odelram 1964) and in the intestine (Johnson and Hanson 1966). By exclusion of other possible causes, this reaction has been attributed to an increased myogenic activity in the precapillary sphincters elicited locally by the raised transmural pressure (cf. Folkow 1964). Thus a predominant precapillary resistance decrease, induced by the hypercapnia, might raise the transmural pressure at the sphincter level, eliciting an increased myogenic activity. The finding of a largely preserved venous-arteriolar response during hypercapnia, indicating that myogenic reactivity of the resistance vessels is still present, indirectly favours this alternative. However it seems likely that both the above mentioned tone-increasing mechanisms i.e. a lowered concentration of vasodilator metabolites and a raised transmural pressure at the precapillary sphincter level, cooperate to produce a reduced CFC during hypercapnia.

In summary during hypoxia there is an impaired protection of the intestinal capillary bed against pressure changes, since the precapillary resistance vessels and sphincters dilate and no longer respond with the characteristic autoregulatory adjustments to e.g. pressure changes, normally displayed by the intestinal vessels. Such a deterioration of the normal vascular responses might in part explain the increase of the intestinal volume, often noticed during hypoxia, reflecting an outward filtration at the capillary level. Conversely during hypercapnia the capillary bed remains to some extent protected against the augmented local pressure and blood flow in that the increased flow passes through a somewhat reduced capillary surface area at a relatively stable capillary pres[sure]. In this situation, therefor[e] the autoregulatory adjustments are much better preserved.

In this connection the possibility of a redistribution of the blood flow between different tissue compartments of the intestinal wall should be considered. The possible occurrence of such redistribution would not per se invalidate the conclusions already drawn. However if capillary permeability or density differs significantly in the separate tissue compartments the CFC decrease during hypercapnia might partly be explained as due to redistribution of the flow into compartments characterized by a less dense capillary network. Although this is a priori not very probable studies are in progress in which the local blood flow distribution can be studied by a special isotope technique (Lundgren 1968).

Whichever the case the present results may have some bearing on the problem

concerning the factor (or factors) responsible for intestinal hyperemia during digestion. It is prerequisite for any vasodilator agent, considered to be mainly responsible for functional hyperemia in a tissue, that it can produce changes that closely mimick the vascular pattern induced during normal functional hyperemia (*cf* Kjellmer 1965). It can be supposed, and has been repeatedly observed, that in this situation not only the resistance vessels but also the sphincters relax, thereby facilitating the exchange of substances across the capillary walls. However such a vascular pattern, typical of most forms of functional hyperemia in vascular tissues differs considerably from that observed during hypercapnia. Consequently it seems unlikely that a locally increased CO_2 tension, accompanied by a reduced pH in tissue and blood, should play any major role in eliciting intestinal vasodilatation during digestive work. During hypoxia on the other hand, the vascular pattern induced seems to correspond far better with that predicted to occur during digestion. Thus, from this particular point of view local hypoxia might be involved in functional hyperemia in the intestine. However since the P_{O_2} of the arterial blood has to be reduced quite markedly in order to obtain any appreciable intestinal vasodilatation, it is for this reason very unlikely that a lowered oxygen tension should be of any major physiological significance in this respect.

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References

ALEXANDER, R. S. The participation of the venomotor system in pressor reflexes. *Circulat. Res.* 1954. *2*. 405—409.

ASTRUP, P. A. A simple electromagnetic technique for the determination of carbon dioxide tension in blood and plasma, total content of carbon dioxide in plasma and bicarbonate content in "separated" plasma at fixed carbon dioxide tension (40 mm Hg). *Scand. J. clin. Lab. Invest.* 1956. *8*. 33—43.

BEAN, J. W. and M. M. SIDKY. Effect of low O_2 on intestinal blood flow, tonus and motility. *Amer. J. Physiol.* 1957. *189*. 3. 541—547.

BERNTHAL, T. and F. J. SCHWIND. A comparison in intestine and leg of the reflex vascular response to carotid-aortic chemoreceptor stimulation. *Amer. J. Physiol.* 1945. *143*. 361—372.

BRADLEY, S. E. *Handbook of Physiology* 1963. Sect. 2. Circulation. Vol. 2. 1387—1438.

BRICKNER, E. W., E. G. DOWDS, B. WILLITT and E. SELKURT. Mesenteric blood flow as influenced by progressive hypercapnia. *Amer. J. Physiol.* 1956. *184*. 275—281.

EPSTEIN, R. M., H. O. WHEELER, M. J. FRUMIN, D. V. HABIF, G. M. PAPPER and S. BRADLEY. The effect of hypercapnia on estimated hepatic blood flow, circulating splanchnic blood volume and hepatic sulfobromophthalein decrease during general anesthesia in man. *J. clin. Invest.* 1961. *40*. 592—698.

FOLKOW, B., O. LUNDGREN and I. WALLENTIN. Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat. *Acta physiol. scand.* 1963. *57*. 270—283.

FOLKOW, B., Autoregulation in muscle and skin. *Circulat. Res.* 1964. 14—15. Suppl. I. I—19—24.

GRIM, E. The flow of blood in the mesenteric vessels. *Handbook of Physiology* 1963. Sec. 2. Circulation. 2. 1439—1456.

JOHNSON, P. C., Myogenic natur of increase in intestinal vascular resistance ith enous pressure elevation. *Cir Res.* 1959. *7.* 992—999.

JOHNSON, P. C. and K. M. H sow, Capillary filtration in the small intestine of the dog. *Circulat. Res.* 1966. *19.* 766—773.

JOHNSON, P. C., Effect of enous pressure on mean capillary pressure and vascular resistance in the intestine. *Circulat. Res.* 1965. *16.* 294—300.

KJELLMER, L., Studies on exercise hyperemia. *Act physiol. scand.* 1965. Suppl. 244.

LANDIS, E. M. and J. R. PAPPENHEIMER, Exchange of substances through the capillary walls. *H ndbook of Physiology* 1963. Sect. 2. Circulation. 2. 961—1034.

LUNDGREN, O. *Studies on blood flow distribution and countercurrent exchange in the small intestine. Acta physiol. sc d.* 1967. Suppl. 303.

MACDOWALL, R. J. S., The effect of carbon dioxide on the circulation. Part. 1. *J. Physiol.* (Lond.) 1930. *70.* 301.

MELLANDER, S. B. ÖBERG and H. ODELRAM, Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Act physiol. scand.* 1964. *61.* 34—48.

SHORT, M. M. and J. W. BEAN, Local and general alterations of blood CO_2 and influence of intestinal motility in regulation of intestinal blood flow. *Amer. J. Physiol.* 1951. *167.* 413—423.

WALLENTIN, I. Studies on intestinal circulation. *Acta physiol. sc nd.* 1967. Suppl. 279.

Acta physiol. scand. 1968. 74. 552—567

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Mode of Binding of Histamine and Some Other Biogenic Amines to a Protamine-Heparin Complex In Vitro

by

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Abstract

ÅBORG, C. H. and B. UVNÄS. *Mode of binding of histamine and some other biogenic amines to a protamine-heparin complex in vitro.* Acta physiol. scand. 1968. 74. 552—567.

Protamine and heparin form a water-insoluble complex (PHC) at pH 7. This complex has the ability to bind sodium, histamine and monoamines electrostatically. Titrations have suggested the PHC to be formed by a linkage between the guanidino groups of the protamine and the sulphuric acid and most of the carboxylic acid groups of the heparin. The cationic binding properties of the PHC may be assigned to the terminal COO$^-$-group of the protamine polypeptide chain. The qualitative similarities in the cation binding properties of the PHC and rat mast cell granules are discussed, with special reference to the mechanism of histamine storage in the mast cell granules.

The ability of heparin to form a complex with histamine has been reported by several authors (Werle and Amann 1955, Sanyal and West 1959, Küttner 1961, Kobayashi 1962). The complex is stable in water especially at acid pH (e.g. in 1 % acetic acid or 0.01 N HCl), but it breaks down in the presence of cations and it is completely dissociated in 0.9 % NaCl solution.

The high content of heparin in the basophil mast cell granules has led to the theory that histamine (and 5-HT) is stored in the granules, electrostatically linked to this acid polysaccharide (for references see Keller 1958, Green and Day 1960, Kobayashi 1962).

The sulphuric acid groups of the heparin are essential for the formation of the heparin-histamine complex, desulphated heparin lacking complex forming capacity (Kobayashi 1962). In the mast cell granules the heparin is present as part of a heparin-protein complex. The protein is reported to be basic with a high arginine content (Benditt and Lagunoff 1964). Gel-filtration (Sephadex G 100) suggests a molecular weight around 5000 or multiples thereof (Bergqvist and Uvnäs, to be published). It is questionable therefore whether the granule heparin has any sulphuric acid groups available for histamine binding, since these groups are probably

involved in the protein-heparin linkage. In fact, recent observations indicate carboxyl groups, and not sulphuric acid groups to be the most likely ionic binding sites for the histamine in the mast cell granules (Åborg, Novotný and Uvnäs 1967). Unfortunately those studies did not allow a more precise localization of the histamine-binding carboxyl groups. Whether they belonged to the protein or to the heparin part of the granule complex (or to both) had to be left for future investigation.

Clupein—a protamine from herring—is a low molecular weight, basic polypeptide with a high content of arginine. This basic protein forms a complex with heparin by coupling to the sulphuric acid and carboxyl groups of the polysaccharide (Fig. 1). Like the protein-heparin complex in the rat mast cell granules, the clupein-heparin complex is insoluble in water but dissociates in salt solutions of sufficient concentrations. The apparent similarities in composition and physico-chemical behaviour of the two protein heparin complexes encouraged us to use the protamine-heparin complex (PHC) for model studies *in vitro* with the principal object of localizing possible binding sites for histamine in the complex.

The studies showed that histamine and other biogenic amines are ionically linked to carboxyl groups, most probably in the protamine part of the PHC.

Experimental Procedures and Materials

Preparation of the protamine-heparin sample

About 0.5 g of sodium heparinate was dissolved in 50 ml of 0.9 % sodium chloride solution, pH 6.8. About 0.5 g of protamine sulphate was dissolved in 25 ml of 0.9 % sodium chloride solution and adjusted to pH 7 with NaOH. Whilst stirring, protamine sulphate solution was added to a large excess of sodium heparinate solution. The milky precipitate formed was centrifuged at 2500×g for 20 min. After washing four times with 75 ml *aq. dest.* the precipitate was stored in *aq. dest.* 4 C. A homogenous suspension of the precipitat contained 3.2 mg protamine-heparinat per 100 µl.

Preparation of resins

The columns used were 170×10 mm. Dowex 50 W X8, 25—50 mesh, was treated with 500 ml of 1.0 M HCl and then washed with deionized water until the pH was constan (pH 6—7). Dowex 2 X8, 20—50 mesh, was treated with 500 ml of 1.0 NaOH and then washed with deionized water until the pH was constant (pH 6—7). The absence of Cl was checked using $AgNO_3$ dissolved dilute HNO

Preparation of other material

H_2SO_4 0.05 and 0.1 M Na_2SO_4 were passed through Dowex 50 W X8.

Heparinic acid 500 mg of sodium heparinate dissolved 100 ml of deionized ter was passed through Dowe 50 W X8.

Protamine base Protamine sulphate sol tion (2.6 g dissolved in 100 ml of deionized water) as passed through Dowex 2 X8 and immediately titrated with HCl or with heparinic acid.

Phenylethylam base 20 mM phenylethylamine-hydrochloride sol tion was passed through Dowex 2 X8. After adjusting pH 7 with 0.1 N H_2SO_4, the solution was diluted to 10 mM ith deionized er

5-HT base T 135 mg of 5-HT hydrogen oxalate was added 35 mg of (a OH) dissolved in 25 ml of deionized w ter slightly less than the quantity required for otal precipitation (calculated to be 37 mg). After immediate neutralization to pH 7 w th 0.2 N H_2SO_4, the mixture was diluted to 10 mM with deionized w er nd he precipita removed by centrifugation.

The amine bases (histamine adrenaline and noradrenaline) were dissolved or suspended deionized w er and adjusted t pH 7 with 0.1 N H_2SO_4.

Oxidation impurities of adrenaline and noradrenaline were removed by shaking with activated charcoal.

Upt ke studie

Uptake f sod m Plastic tubes with stoppers were weighed. To each tube was added 2 ml of sodium phosphat buffer (pH 7) with admixture of ^{22}NaCl (conc see below) followed by 100 µl of the protamine-heparinate suspension (3.2 mg dry weight) After 10 min incubation, the samples were centrifuged for 20 mi at 2500×g The lear supernatants were removed by suction except for 1—2 drops which were left in the tubes. Each tube was reweighed, and the total olume of the contaminating radioacti fluid was determined by deducting the weight of the protamine-heparinate (3.2 mg) nd correcting for the dilution. The radioactivity i the t bes was then measured in Philips P.B. 41119 type 03 scintillation detector model 41180 (well-type crystal) Since the reduction in the radioactivity of the incubation medium due to the ptake of sodium into the PHC could be neglected, the uptake of sodium into the PHC could be calculated from the total radioactivity of the contaminating suspension fluid.

The pH of the incubation fluid was aried between pH 4 and pH 7 by mixing solutions of 10 mM NaH_2PO and 5 mM Na_2HPO nd abov pH 7 by adding 10mM NaOH to the Na_2HPO_4 solution.

To give suitable level of radioactivity (about 30,000 cpm per sample) 0.1 ml of ^{22}NaCl solution was added to 10 ml of incubation buffer

Uptak of C labelled am s. The procedures were the same as sed for the sodium uptake studies but after weighing the tubes at the end of the incubation, 0.5 ml of 1.0 N N OH was addd to dissolv the protamine-heparinat 200 µl from each tube was pipetted into 15 ml of scintillation fluid and the radioactivity was measured i a Packard Tri-Carb liquid scintillation spectromet model 2002. The liquid scintillation fluid contained 0.4 % 2,5-diphenyloxazole and 0.01 % 1.4-bis-2(4-methyl-5-phenyloxazolyl)-benzene in a mixture of equal olumes of toluene and ethylene glycol monoethyl ether

Calculations of the ptake were made as described in the sodium uptake experiments (*vide supr*)

All procedures (the uptak (and release) tudies prior to measuring the radioactivity were performed at 0 C.

The amounts f radioactiv material added in the uptake and release studies were ^{22}Na, about 2 µc/7 ml ^{14}C amines, 0.2 µc (for noradrenaline about 0.02 µc)/2 ml

Hist mi e was determined fluorimetrically using slight modification of the method of Shore Burkhalter and Cohn (1959)

Materials

Histamine (ri g-2 C)d hyd ochloride Sp.act. 30.5 mc/mM The Radiochemical Centre England.

DL-ad nali (arbi ol C) DL-bitart at Specific activity 7.3 mc/mM, 21.9 µc/mg The Radiochemical Centre England.

DL-norad enaline (arbinol-^{14}C) DL-bitart te Specific activity 41.2 mc/mM The Radiochemical Centre England.

Phenylethylami 1 C HCl Specific activity 2.0 mc/mM New England Nuclear Corp USA.

5-hyd xytryptami 2 C hyd ogen xalat Specific activity 4.46 mc/mM. New England Nuclear Corp., USA

^{22}NaCl. Sodium chloride ^{22}Na>1 mc/mg Philips-DUPHAR, Holland.

H parin. (B.P.63) Activity 138.7 U/mg calculated as the dried base AB Vitrum Sweden

Protami sulphate AB V trum, Sweden

Results

Part I

Uptake of sodium

When suspended in sodium phosphate buffer pH 7 at 0° C the PHC showed considerable ability to take p sodium (Fig 1) The uptake rose as the concentration of sodium in the suspension med um was increased approach ng a maximal level of around 125 mµmoles/mg PHC t a sodium concentration of 100 mM

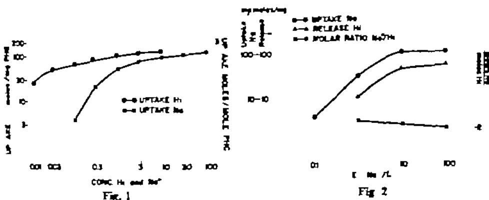


Fig. 1 Uptake of histamine and sodium by PHC. Assumed mol.wt. of PHC 16000. Note the two uptake curves approach the same maximal uptake level of 2 moles per mole PHC.

Fig. 2. Exchange between sodium and histamine in PHC previously suspended in 10 mM histamine at pH 7 Exchange experiment performed in sodium phosphate buffer pH 7

Uptake of histamine

Histamine has a greater affinity for th PHC than sodium (Fig. 1) the uptake curve being shifted to the left and approaching a maximal level of around 125 mμmoles/mg PHC already at a 10 mM histamine concentration in the suspension medium. (Due to dissolution of the PHC, uptake studies could not be performed in histamine concentrations above 30 mM.)

Comments

The fact that, calculated on a molar basis, the maximal uptake of sodium and of histamine by the PHC is approximately the same is in accordance with th assumption of an identical mode of binding of the two ions. The different courses of the uptake curves indicate differences in the affinities of the two ions for the binding sites. Judging from the concentrations required to give half maximum uptake, the affinity of histamine for th PHC is about 30 times higher than that of sodium.

The PHC goes into sol tion when the NaCl concentration reaches 1 7 M. Dissolution of the complex also occurs when the histamine concentration exceeds 30 mM. A possible explanation of this phenomenon is that there is a break n the linkage between the NH -groups of the protamine and the acid groups of the heparin, with an ultimate dissociation of the PHC into heparin and protamine (see Fig 14)

Release f histamin with sodium

If histamine and sod'um were bound to the same ionic sites in the PHC, a competitive exchange should occur at the binding sites between sodium and histamine. In order to demonstrate such competition, PHC was first suspended in a histamine con centration sufficient to result n high ptake of histamine (10 mM mixture of hista

mine base and histamine dihydrochloride yielding pH 7) After centrifugation, the PHC was washed three times in deionized water and then suspended in various concentrations of sodium phosphate buffer pH 7 containing ^{22}NaCl

The uptake of sodium was calculated from the radioactivity of the precipitate and the release of histamine was determined chemically Both procedures are described under "Methods"

The presence of sodium in the suspension medium led to the expected exchange between this sodium and the histamine in the PHC. The almost parallel course of the sodium uptake and the histamine release curves (Fig. 2) was in accordance with a competition of the two ions for the same sites in the PHC.

Comments

The molar ratio between the uptake of sodium and the release of histamine was around 2 at high sodium concentrations (100 mM) but increased to around 3 at low sodium concentrations (1 mM) Since both histamine and sodium reach the same maximal uptake values—around 125 mμmoles per mg PHC (Fig. 1)—and since histamine is monovalent at pH 7 (according to Long, 1961 the pKa values of histamine are 5.97 and 9.8) at high sodium ion concentrations the molar exchange ratio between sodium and histamine would be expected to approach 1 The reason why higher molar exchange ratios were usually observed will be discussed below (p. 558)

Influence of pH on the binding of sodium and histamine

For ionic binding of cations the PHC contains two different kinds of anions, those derived from the rather strong acids—$NHSO_2OH$ and—OSO_2OH in the heparin and those derived from—COOH in both heparin and protamine (Fig. 12) To determine whether COO groups or SO_2O-groups were responsible for binding Na and Hi the experiments described below were performed

Histamine PHC was incubated in 1 mM histamine (base + dihydrochloride) pH 7 containing C-histamine After one washing with water the PHC was suspended in 50 ml of deionized water and acidified stepwise by the addition of HCl. At various pHs 2 ml samples were removed for determination of the histamine content of the supernatant (calculated from its radioactivity)



Fig 3 Influence of pH on histamine binding in PHC previously suspended in 1 mM histamine pH 7

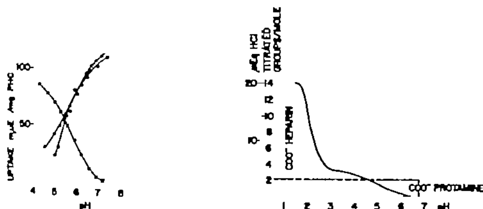


Fig. 4. Influence of pH on the uptak of sodium and hydrogen by PHC
●——● Uptake Na ; ■——■ Uptake H ;
◄——◄ Uptake Na constructed from H uptake.

Fig 4 a. Titration of PHC with 5 N HCl t 0 C. (12 out of the expected 14 COO− groups in the heparin demonstrated)

The binding capacity proved to be pH-dependent, falling rapidly between pH 7 and pH 5 and being abolished below pH 4.5 (Fig 3)

Sodium. The uptake of sodium by the PHC took place over a similar narrow pH range on incubation in sodium phosphate buffers of constant normality (10 meq Na /l) but of different pHs. The uptake at pH 7 was observed to be around 125 mueq per mg PHC but at pH 5 only 30 mueq (Fig 4)

Hydrogen. The uptake of hydrogen by the PHC was determined by titration with HCl. The titration curve forms a rather good mirror image of the "found" sodium uptake curve. This is further corroborated by th close agreement between the "found" sodium uptake curve and the theoretical' sodi m uptake curve constructed from the titration curve, suggesting an equimolar exchange between sodium nd hydrogen ions (Fig. 4)

Comments

At room temperature titrations with HCl could not be carried out below pH 4—4.5 At 22° C physico-chemical changes (gel formation) occurred in the PHC. T tration to low pH is possible at 0° C indicating an increased stability of the complex t this temperature (Fig. 4 a)

Facto infl encing the mol hang atio between sod m nd histamin

The pH curves for the binding of sodium, histamine and hydrogen (Fig 3 and 4) indicate binding of these cations to weak acid groups in the PHC. Assuming a competition for the same ionic sites, w did not expect to find, at pH 7 a considerable variation in the molar exchange ratio between sodium uptak and histamine release.

mine base and histamine dihydrochloride yielding pH 7). After centrifugation, the PHC was washed three times in deionized water and then suspended in various concentrations of sodium phosphate buffer pH 7 containing ^{22}NaCl.

The uptake of sodium was calculated from the radioactivity of the precipitate and the release of histamine was determined chemically. Both procedures are described under "Methods".

The presence of sodium in the suspension medium led to the expected exchange between this sodium and the histamine in the PHC. The almost parallel course of the sodium uptake and the histamine release curves (Fig. ?) was in accordance with a competition of the two ions for the same sites in the PHC.

Comments

The molar ratio between the uptake of sodium and the release of histamine was around 2 at high sodium concentrations (100 mM) but increased to around 3 at low sodium concentrations (1 mM). Since both histamine and sodium reach the same maximal uptake values—around 125 mμmoles per mg PHC (Fig. 1)—and since histamine is monovalent at pH 7 (according to Long, 1961 the pKa values of histamine are 5.97 and 9.8) at high sodium ion concentrations the molar exchange ratio between sodium and histamine would be expected to approach 1. The reason why higher molar exchange ratios were usually observed will be discussed below (p. 558).

Influence of pH on the binding of sodium and histamine

r ionic binding of cations the PHC contains two different kinds of anions: those derived from the rather strong acids—NHSO$_2$OH and—OSO$_2$OH in the heparin and those derived from—COOH in both heparin and protamine (Fig. 12). To determine whether COO groups or SO$_2$O -groups were responsible for binding Na and Hi the experiments described below were performed.

Histamine. PHC was incubated in 1 mM histamine (base + dihydrochloride) pH 7 containing ^{14}C-histamine. After one washing with water the PHC was suspended in 30 ml of deionized water and acidified stepwise by the addition of HCl. At various pHs 2 ml samples were removed for determination of the histamine content of the supernatant (calculated from radioactivity).



Fig. 3. Influence of pH on histamine binding in PHC previously suspended in 1 mM histamine pH 7.

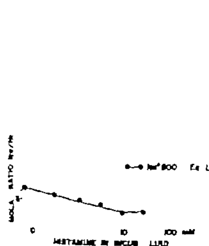


Fig. 5

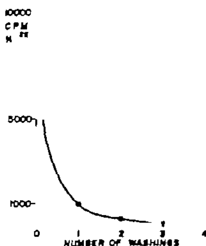


Fig 6

Fig. 5 Influence of saturation of PHC with histamine on the molar exchange ratio N /HI

Fig. 6 Release of sodium ions from PHC by repeated washing in deionized water

As expected from its lower affinity for the PHC (Fig. 1) sod um losses on washing sodium-containing PHC were considerably greater than the corresponding losses of histamine. A few washings sufficed to completely deplete PHC of its sodium (Fig. 6)

Conclusions. The observations reported above concerning the ptake and release of sodium, histamine and hydrogen ions in a protamine-heparin complex, the competition between these cations for binding sites in this complex as well as the strong influence of pH on the b nding capacity of the complex all support the theory of an electrostatic linkage of these cations to weak acid groups in the complex

The only weak acid groups in the PHC available for ionic binding are carboxyl groups, which are to be found in both the protamine and heparin component of the complex. Part II of this paper will deal with the localization within the protamine heparin complex of th cation binding sites and with some quantitati e aspects of the binding.

Part II

Quantitative pect f th bind ng f odium and histam ne in the prot mi hepa rin complex (PHC)

Cl pein—th protamine used in the present study—has a high content of argi ine 90 of the total nitrogen being contained in arginine This amino acid frequently occurs in tetrapeptides which are separated by dipeptides of monoam no acids The polypeptid is believed to ha e only two free groups one t each end of the open-peptide hain, one carboxyl nd one amino (or imino) group (Fig. 7

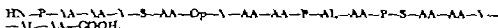


Fig 7 Clupein.
AA = Arginyl-Arginine, AL = Alanine S = Serine P = Proline Op = Oxyproline, V = Valine

Molecular weight of clupein. 500 mg of clupein sulphate (dried *in vacuo* over P_2O_5) was dissolved in 15 ml of deionized water and titrated with 5 N NaOH to pH 11 The titration curve (*Fig 8*) shows two points of inflexion—at pH 5.5 and pH 10. On the assumption that the titration curve reflects the release of hydrogen ions from *one* terminal COOH and *one* terminal NH_3 (or NH_2) group per clupein molecule (as suggested *e.g* by Linderström-Lang 1935 and by Felix and Mager 1937) the amount of NaOH consumed—about 100 μeq per terminal group (Fig. 8)—corresponds to a molecular weight of the clupein sulphate of about 5000 (or multiples thereof) a reasonable value to judge from reports in the literature (Linderström-Lang 1935 Ando *et al* 1962) In the calculations below 5000 was used as the probable mol.wt. of our the clupein sulphate Since the sulphate groups correspond to about 20 per cent of the clupein molecule, the mol weight of the clupein base will be around 4000.

Number of guanidino groups in clupein Clupein base was prepared by passing 15 ml of clupein sulphate solution (2.6 g/100 ml *aq.d st*) through a Dowex 2 X8 column and immediately titrated with 5 N HCl. Assuming a mol.wt. of 5000 (*vide supra*) 22 eq of HCl were required per mole of clupein to change the pH from 12.6 10 (Fig 9) The results agree with the presence of 22 free guanidino groups, as proposed by Linderström-Lang 1935. In Fig 12 the titration curve between pH 10 and pH 3 showing one free amino (or imino) and one free carboxyl group is constructed from Fig 9 (The curve between pH 10—3 is taken from Fig 8)

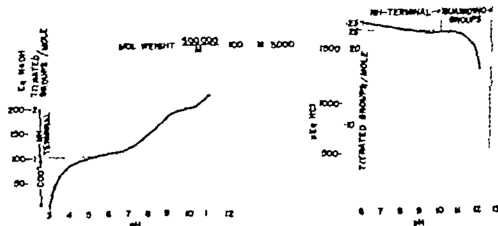
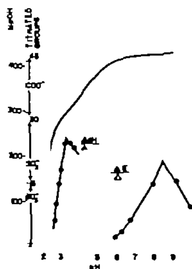


Fig. 8. Titration of 500 mg protamine sulpha w h N OH
Fig 9 Titration of protamine base th HCl Assumed mol w 4000


Fig. 10

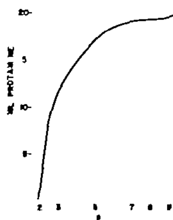

Fig. 11

Fig. 10. Titration of heparinic acid with NaOH

Fig. 11. Titration of heparinic acid with protamine base

Number of acid groups in heparinic acid. The molar weight of sodium heparinate was assumed to be 9000 (7500—12,000 according to Vitrum). Heparinic acid was prepared by passing a solution of sodium heparinate (500 mg (dried *in vacuo* over P_2O_5)/100 ml) through a Dowex 50 W X8 column. 17.0 ml heparinic acid so obtained was titrated with 5 N NaOH. About 44 eq per mole heparin were required to raise the pH from 1.95 to 7 and further 0.6 eq to increase the pH from 7 to about 9 indicating the presence of carboxyl groups still undissociated at pH 7 (Fig. 10).

Molar ratio between protamine and heparin in the PHC. Heparinic acid and clupein base were prepared by ion exchange chromatography (on Dowex 50 W X8 and Dowex 2 X8 respectively). 18.5 ml of heparinic acid solution (corresponding to 5 mg/ml Na-heparinate) required 19.1 ml of clupein base (corresponding to 5 mg/ml clupein sulphate) to change the pH from 1.9 to 7 (Fig. 11). The molar ratio suggested from the titration curve is around 2 (1.86).

Gross structure of the PHC. To judge from the titration results given above the composition of our PHC can be assumed to correspond to the gross structure drawn in Fig. 12. This figure is composed from the results presented in Fig. 9 and 10. At pHs around 7, 2 *moles* of protamine base can be expected to bind *1 mol* of heparinic acid through linkages between 44 (2 × 22) guanidino groups in the protamine and 44 acid groups (2 × 15 SO and about 14 COO groups) in the heparinic acid. The remaining carboxyl groups of the heparin should still be undissociated at neutral pH. The free groups of the PHC should be confined to one COO and one NH (or NH) group (the two end groups) of the protamine.

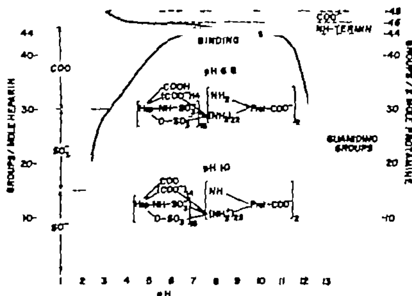


Fig. 12 Proposed gross structure of the PHC composed from data in Fig. 9 and 10 (assuming the mol.wt of heparin be 9000)

The following titration experiment also supports the gross formula in Fig 12 PHC was saturated with Na ions by suspending 241 mg of PHC in 10 ml of N NaCl. The pH of this suspension medium fell from 6.8 to 5.5 To readjust to pH 6.8 very little NaOH was required (Fig 13) supporting a dipolar binding of the NaCl to the COO and NH_3 groups of the PHC (Fig 14) To raise the pH to 10 required more than 40 µeq of NaOH corresponding to 3 acid groups per mole of complex. Possibly previously undissociated carboxyl groups of the heparin were dissociated, while the dissocia-

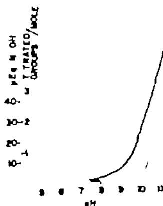


Fig 13. Titration of PHC with NaOH
IN 10 ml N NaCl
——— IN 10 ml AQ DEST
ONLY 10 ml N NaCl

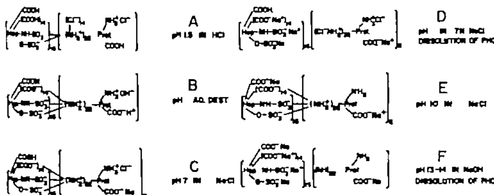


Fig. 14. Suggested dipolar binding of NaCl to the protamine end groups of PHC.

tion of the imino group of the protamine was suppressed with a consequent release of H ions (Fig. 14)

The above results support a mol.wt. of the PHC of around 16,000 (cl pein base 4000, heparinic acid 8000)

The sodium and histamine binding sit of the PHC According to Fig 12, the COO end group of the protamine should be the only free anion in the PHC at pH 7 Consequently the maximal uptake of cations by the protamine part of the complex (as depicted in Fig. 12) should correspond to 2 moles per mole of the complex. Fig 1 shows the uptake curves for sodium and histamine. Both curves showed a maximal uptake of around 125 mμeq/mg of the complex. Assuming the mol.wt. of the PHC to be around 16,000 (see p 560) the maximal uptake of sodium and histamine approaches 2 moles per mol of PHC, a val e in good agreement with the suggested gross formula shown in Fig 12.

Qualitative support for th theory that the protamine carboxyl groups are cationic binding sites was obtained from observations on a protamine heparin complex in which the carboxyl end groups of th protamine component were reduced to primary alcohol groups.

Protamine sulphate was esterified in methyl alcohol and the methyl ester then reduced with lithium borohydride according to Chibnall and Rees 1958. The COOH of the protamine is thus changed into CH_2OH The reduced protamine was precipitated in acetone/ether (1 1) and the precipitate was washed twice in acetone and once in ether After drying, the precipitate was dissolved in 0.9 % NaCl, filtered and adjusted t pH 7 with NaOH. A precipitate formed on the addition of sodi m heparinate in 0 9 % NaCl. This precipitate was washed twice in ater and then used for sodium ptake studies using the method described for PHC

Treatment with lithium boroh dride 0.45 M markedly reduced th ptak of sodium by the precipitat t pH 7 (Fig. 15) with 1 4 M borohydride t was bolished. The ptake of sod um by the red ced PHC bove pH 7 is presumably due to the cation bind ng capac ty of d sociated carboxyl groups of the heparin.

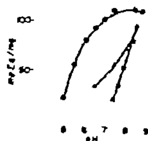


Fig. 15

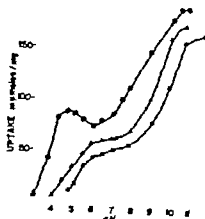


Fig. 16

Fig. 15. Uptake of Na ions in PHC with reduced carboxyl groups (COOH → CH OH)
●——●PHC with intact COO-
◄——◄PHC treated with 0.45 M $LiBH_4$
■——■PHC treated with 1.4 M $LiBH_4$

Fig. 16. Influence of pH on the uptake of sodium, 2-phenylethylamine and histamine in PHC.
●——●9.5 mM histamine
►——►9.5 mM phenylethylamine
■——■9.5 mM sodium

The uptake of cations by the non-reduced PHC increased rapidly above pH 7 Fig. 16. At pH 10—11 the uptake had increased two- to threefold above the pH value. The increased uptake of cations at alkaline pH can presumably be ascribed to unmasking of undissociated carboxyl groups in the heparin and such groups as were previously linked to guanidino groups of the protamine (see Fig. 19)

The curve showing the uptake of histamine at various pHs differed from that of sodium and the monoamine 2-phenylethylamine in that it showed a peak between pH 4 and 5. This peak is probably due to the fact that below pH 7 histamine becomes divalent, whereby the number of ionic binding sites increases but the dissociation of the carboxyl groups of the protamine diminishes. Below pH 7 the binding of histamine to PHC will depend on the balance between these two processes acting in opposition.

Binding of other biogenic amines to PHC. As expected, PHC also binds other biogenic amines as well as histamine. To judge from the uptake curves in Fig. 17 there seems to be little difference in the maximal uptake values at pH 7 for sodium, histamine, adrenaline and noradrenaline. They all show a maximal uptake corresponding to about 2 moles per mol of PHC. Thus they all seem to be linked to the PHC via their amino groups. To judge from the slopes of the uptake curves, the amines differ somewhat in their affinities for the PHC. The reason for these differences in affinity has not been investigated. From the concentrations needed to produce half maximal uptake the following affinity sequence is obtained

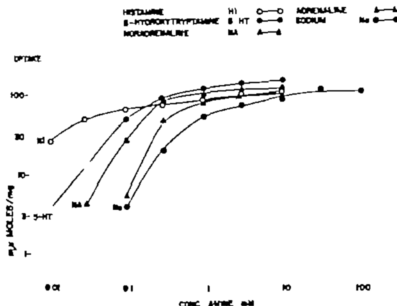


Fig. 17 Uptake of sodium and various biogenic amines by PHC.
Hi = histamine 5-HT = 5-hydroxytryptamine; N = sodium A = adrenaline NA = noradrenaline

histamine > 5-hydroxytryptamine > noradrenaline > adrenaline > sodium

It should be stressed that the uptake values at concentrations around 0.1 mM and less are unreliable due to insufficient buffer capacity of the amine solutions. The acidity of our deionized water may suffice to acidify the suspension medium and thus reduce the uptake.

Discussion

Protamine and heparin form a water insoluble complex at pH 7 The present experiments have demonstrated the ability of this complex (PHC) to bind sodium, histamine and various monoamines electrostatically

Titrations of free acid and alkaline groups in protamine base, heparinic acid, and in the PHC suggest the PHC to be formed by the linkage of one heparin and two protamine molecules. The guanidino groups of the protamine are linked to the sulphuric acid groups and most of the carboxylic acid groups of the heparin, the few remaining carboxylic acid groups of the heparin being undissociated at pH 7 (Fig 12) At this pH the only dissociated groups of the PHC seem to be the terminal NH and COO groups of the protamine component of the complex Since the PHC contains 2 moles of protamine per mole of heparin, each mole of PHC has two equivalents of COO-groups and thus should be able to bind two equivalents of cations.

The slopes of the pH curves for the uptake and release of cations in the PHC indicate a cation binding to weak acid groups. The only available groups with such properties are carboxyl groups in the protamine.

The maximal cation binding capacity of the PHC corresponds to an uptake of 2 moles per mole of PHC, a saturation level to be expected if the terminal COO groups of the protamine were the only cation binding sites of the PHC.

Further support for the above theory is the fact that a PHC deprived of terminal carboxyl groups showed a reduced ability to bind cations at pH 7 or no ability at all.

The unspecificity of the binding sites of the PHC is illustrated by the fact that the complex takes up not only sodium and histamine but also several monoamines. The maximal uptake of these amines corresponds to 2 moles per mole of PHC, indicating a binding between the NH_3 or NH_2-groups of the amines and the terminal COO groups of the protamine.

Above pH 7 the binding capacity of the PHC increases rapidly. This increase may be explained as being due to more COO groups becoming available by dissociation of carboxyl groups in the heparin part of the complex (see Fig. 12).

A protein-heparin complex is the main constituent of the granules in rat peritoneal mast cells. This protein-heparin complex is insoluble in water at pH 7 but it dissolves in salt solutions (*e.g.* 1 N NaCl) or in alkaline solutions (10 mM Na pH 9). On electrophoresis the dissolved protein-heparin complex separates into heparin and a basic protein (I.P. 9—10, mol.wt. about 5000, Bergqvist and Uvnäs to be published).

In vivo the mast cell granules store histamine and 5-HT in considerable amounts, the content of histamine corresponding to a 0.4 M concentration in the granule. *In vitro* the granules have been shown to release their histamine when exposed to cations (*e.g.* sodium chloride in the suspension medium). The release is due to an exchange at ionic binding sites between histamine and sodium. The influence of pH on the binding capacity of the granules indicates the binding sites to be COO groups. As was the case with the PHC, the binding capacity rapidly decreases below pH 7 but increases on the alkaline side of neutrality, indicating the unmasking of COO or SO groups above pH 7. The mast cell granules are able to bind not only sodium and histamine but also monoamines, as was shown to be the case for PHC. Likewise sodium, histamine and the monoamines seem to compete for identical ionic sites in the granules.

As is evident from the present observations on the PHC and previous experiments on mast cell granules referred to above (Åborg, Novotny and Uvnäs 1967), the PHC and the mast cell granules show striking similarities in their amine binding properties. In fact, the agreement is qualitatively so close that to us it seems justified to take the observation on the PHC as supporting our previous suggestion that the carboxyl groups of the protein-heparin complex forms the histamine-carrying component of the mast cell granules, and not the sulphate groups of heparin as generally suggested. Quantitatively the mast cell granules have a considerably higher binding capacity for histamine than the PHC ~1000 mµmoles/mg granules, dry weight against 1.5 mµmoles/mg PHC, dry weight. The higher binding capacity of the protein-heparin

complex in the mast cell granules may be due to several factors. The protamine in the PHC contains only monocarboxylic acids the only free COO groups being the terminal groups of the protamine polypeptide chain. On the other hand, the protein of the mast cell protein-heparin complex has been shown to contain dicarboxylic acids as well (Bergqvist and Uvnäs to be published) The presence of a sufficient number of such divalent acids might explain the higher cation binding capacity of the granules.

Furthermore the protein of the mast cell protein-heparin complex is considerably less basic (I.P 9—10) than protamine Consequently COO groups from the heparin might also be available for cation binding Investigations are under way to elucidate further the localization of the histamine binding sites in the protein-heparin complex.

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References

Ådén, C. H J Novotny and B. Uvnäs, Ionic binding of histamine in mast cell granules. *Acta physiol. scand.* 1967 *69* 276—283

Ando, T K. Iwai, S I. Ishii, M. Azegami and Ch. Nakahara, Chemical structure of one component of clupein. *Biochem. Biophys. Acta.* 1962. *56* 628—630.

Benditt E. P and D Lagunoff The mast cell its structure and function. *Progr Allergy* 1964 *8*, 183—223

Chibnall, A. C. and M. W Rees, Studies on the amide and C-terminal residues in proteins. *J Biochem.* 1958, *68* 105—111

Felix, K. and A. Mager, Über Clupein. *Z. Physiol. Chem.* 1937 *249* 111—123

Green J P and M. D v Heparin, 5-hydroxytryptamine and histamine in neoplastic mast cells. *Biochem. Pharmacol.* 1960. *3—4* 190—205.

Keller, R., Zur Bindung von Histamin und Serotonin in den Mastzellen. *Arzneim. Forsch.* 1958, *8* 390—394.

Kobayashi, Y Histamine binding by heparin. *Arch. Biochem.* 1962. *96* 20—27

Kuttner, K *Ionenaustausch biologisch wichtiger Amine an synthetischen und natürlichen Austauschern als Modell für die pharmakologische Histaminliberation* Dissertation. Bern 1961 pp. 77

Linderström Lang K., Some electrochemical properties of a simple protein. *Transactions of the Faraday Soc* 1935. *31* 324—335.

Long, C., *Biochemists Handbook*. London, Spon. 1961 pp. 1192.

Saeki, R. K. and G. B. West The histamine-heparin complex. *J Pharm* 1959 *11* 548—2.

Shore, P A A. Burkhalter and V H Cohn A method for the fluorometric assay of histamine in tissue. *J Pharmacol exp Ther* 1959 *127* 182—186

Weill, E. and R. Amann, Über eine Bindung des Histamins am Heparin. *Naturwissenschaften* 1955 *42*, 383

Acta physiol scand. 1968. 74 568—576

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In Vivo and in Vitro Studies of the Regulation of Palmityl-CoA Synthetase Activity in Rat Liver

By

Mikael Farstad[1]

Received 23 March 1968

Abstract

Farstad, M. *In vivo and in vitro studies of the regulation of palmityl CoA synthetase activity in rat liver* Acta physiol scand 1968. *74* 568—576

An assay system is described to evaluate the palmityl-CoA synthetase activating capacity of particle free supernatant from different sources. With relatively small amounts of tissue the activating capacity was proportional to the amount of supernatant present. The palmityl-CoA synthetase activity in whole homogenates of rat liver was reduced after feeding with sucrose and increased after fasting The palmityl-CoA synthetase activating capacity of rat liver supernatant was also significantly increased after fasting $MgCl_2$ increased the inactivation of microsomal palmityl-CoA synthetase in vitro, NaCl and NaF reduced the inactivation, and ATP prevented any inactivation. $CaCl_2$ but not KCl, also reduced the inactivation in vitro. Inactivated microsomal palmityl-CoA synthetase was partially re-activated by incubation with particle-free supernatant and ATP The results suggest that two forms of palmityl-CoA synthetase may be interconvertible through reversible phosphorylation, catalyzed by two different enzymes, i.e. the supernatant activating factor and phosphoprotein phosphatase.

We have previously shown that the particle-bound palmityl-CoA synthetase (Acid CoA ligase AMP EC 3.. 1 3) in organs from rodents is activated by preincubation with particle-free supernatant from organs of the same animal or of other rodents (Farstad 1967 a) The activation is dependent on ATP and Mg^{+} in approximately equimolar concentrations (Farstad 1967 a)

The presence of supernatant does not stimulate the formation of either palmityl-CoA or ATP from the known intermediate palmityl AMP supporting the contention that ATP is required for the activation of palmityl-CoA synthetase by supernatant (Farstad 1967 b)

The results have lead to the suggestion that the soluble activating factor in the cytoplasm is an enzyme with properties similar to those of phosphorylase *b* kinase (EC 2.7 1 38.) (Krebs and Fischer 1956 Krebs *et al* 1966) The observation that ATP probably is active at two sites in the activated system gives further support to this hypothesis (Farstad 1967 b

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The present experiments show that fasting and feeding result in differences in the synthetase activating capacity of rat liver supernatant. Results are also reported which show that inactivation of palmityl-CoA synthetase *in vitro* is enhanced in the presence of exogenous Mg^{++} prevented by ATP and is partly reversed by incubation with particle free supernatant and ATP

Materials and methods

Reagents

CoA, ATP and crystalline bovine serum albumin were purchased from Sigma Chemical Company St. Louis, Mo., U.S.A., and DL-carnitine and palmitate from Fluka AG Buchs, Switzerland. DL-CH_3-^3H-Carnitine with specific activity of 75 μC/μmole was prepared according to Bremer and Norum (1967) and diluted with DL-carnitine to specific activity of about 0.6 μC/μmole. Palmitate/albumin solutions were prepared with about 6 moles palmitate per mole albumin. Carnitine palmityltransferase (E.C. 2.3.1) was prepared according to Norum (1964) as previously modified (Farstad et al. 1967)

Animals

For most experiments male rats of local strain were used. Male rats of Wistar strain (Møllegård avlslaboratorium, Havdrup, Denmark) were used for the fasting-feeding experiments. They were adapted for one week in room with syncronized light giving darkness from 7.00 p. m. to 7.30 a. m. All rats were fed on commercially available diet containing 25 per cent carbohydrate, 25 per cent protein and 2.6 per cent fat. One group of rats was given 10 % sucrose as their only food for 56 hrs. "Refed" rats were fasted for 36 hrs, then fed on stock diet for 12 hrs. All rats, except the group fasted for 8 hrs, were killed between 8.00 and 9.00 a. m.

Preparation of whole homogenates and subcellular fractions of rat liver

Rat livers were homogenized in 9 volumes 0.25 M sucrose in Potter Elvehjem homogenizer. Whole homogenates were filtered through glass wool to remove debris. A combined particulate fraction (mitochondria + lysosomes + microsomes) was prepared from the cytoplasmic extract (DeDuve et al. 1955) by centrifugation at 120 000 × g for 30 min. A microsomal fraction was prepared after removal of the mitochondria (12 000 × g for 5 min) by centrifugation at 120 000 × g for 30 min. The final supernatant was collected and was the "supernatant" used. A partially purified activating enzyme was prepared from supernatant by precipitating twice with $(NH_4)_2SO_4$ as previously described (Farstad 1967). The fraction from the second 40—60 % saturation was almost free from palmityl-CoA synthetase and was used for the reactivation studies.

Enzyme assay

Palmityl-CoA synthetase was assayed by the formation of palmitylcarnitine from palmitate, ATP and L-CH_3-^3H-carnitine in the presence of catalytic amounts of CoA and excess amounts of carnitine palmityltransferase as previously described (Farstad et al 1967) In experiments where supernatant was used in combination with particulate fractions, the synthetase activity was corrected for the synthetase contaminating the supernatants.

Results

Assay of the palmityl-CoA synthetase activating capacity of particle-free supernatants

The palmityl-CoA synthetase activation by particle-free supernatants is likely to be the result of an enzymatic activity. Purified palmityl CoA synthetase cannot be obtained at present.

The synthetase activating capacity therefore must be given as a relative increase of a known synthetase activity in an isolated particulate fraction, assayed under standard conditions. An assay system has been worked out to evaluate variations

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Abstract

Farstad M *In vivo and in vitro studies of the regulation of palmityl-CoA synthetase activity in rat liver* Acta physiol. scand 1968. *74* 568—576

A assay system is described evaluating the palmityl-CoA synthetase activating capacity of particle-free supernatant from different sources. With relatively small amounts of these the activating capacity was proportional to the amount of supernatant present. The palmityl-CoA synthetase activity in whole homogenates of rat liver was reduced after feeding with sucrose and increased after fasting The palmityl-CoA synthetase activating capacity of rat liver supernatant was also significantly increased after fasting $MgCl_2$ increased the inactivation of microsomal palmityl-CoA synthetase *in vitro* NaCl and NaF reduced the inactivation and ATP prevented any inactivation. $CaCl_2$ but not KCl, also reduced the inactivation *in vitro*. Inactivated microsomal palmityl-CoA synthetase was partially re-activated by incubation with particle-free supernatant and ATP The results suggest that two forms of palmityl-CoA synthetase may be interconvertible through reversible phosphorylation, catalyzed by different enzymes, i.e. the supernatant activating factor and phosphoprotein phosphatase

We have previously shown that the particle bound palmityl-CoA synthetase (Acid CoA ligase AMP EC 6.2.1.3) in organs from rodents is activated by preincubation with particle free supernatant from organs of the same animal or of other rodents (Farstad 1967 a The activation is dependent on ATP and Mg^{2+} in approximately equimolar concentrations (Farstad 1967 a)

The presence of supernatant does not stimulate the formation of either palmityl-CoA or ATP from the known intermediate palmityl AMP supporting the contention that ATP is required for the activation of palmityl-CoA synthetase by supernatant (Farstad 1967 b)

The results have lead to the suggestion that the soluble activating factor in the cytoplasm is an enzyme with properties similar to those of phosphorylase *b* kinase (EC 2.7.1.38.) (Krebs and Fischer 1956 Krebs *et al* 1966) The observation that ATP probably is active at two sites in the activated system gives further support to this hypothesis (Farstad 1967 b

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TABLE I. The effect of fasting and feeding of different diets on the palmityl-CoA synthetase activity in whole homogenates of rat liver

The palmityl-CoA synthetase activity was assayed as given in the legend to Fig. 1

	Number of rats	Enzyme activity micromoles/g/min Mean ± SD
Fasted 24 hrs, then fed stock diet 12 hrs	7	1.10 ± 0.25
Fed on stock diet	4	1.05 ± 0.43
Fed sucrose 36 hrs	5	0.82 ± 0.19+
Fasted for 8 hrs	5	1.53 ± 0.24++
Fasted for 24 hrs	8	1.90 ± 0.21+++

Significance of difference from the refed group. ($p < 0.05$), ++ ($p < 0.02$) +++ ($p < 0.001$)

As shown in Fig. 1 the activation of palmityl-CoA synthetase increased in proportion to the amount of added supernatant whether various of particulate fraction from the same rat were used (A) or when supernatants from different rats were added to particulate fractions from other rats (B). Fig. 1 B also shows that the activating capacity varied in supernatants from different rats. In all cases the synthetase activity increased with the amount of supernatant up to the amount obtained from approximately 6 mg of liver. With small amounts of particulate fraction, viz. the amount obtained from less than 5 mg of liver, the linearity of the activation existed also with larger amounts of supernatant.

The effect of feeding and fasting on the palmityl-CoA synthetase in whole liver homogenates

Table I shows that the palmityl-CoA synthetase activity was about the same in whole homogenates from liver of rats fed on stock diet and rats fed on stock diet after a period of fasting. Feeding with sucrose as the only food for 36 hrs significantly reduced the palmityl-CoA synthetase activity in whole liver homogenates. The synthetase activity was significantly increased in liver of rats fasted for 8 hrs. The activity was further increased after fasting for 24 hrs, almost doubling the value of the refed group of rats.

The effect of fasting on the palmityl-CoA synthetase activating capacity of particle-free supernatants

As shown in Fig. 2, the synthetase activating capacity was increased in supernatants from liver of fasted rats when compared with fed rats. The difference was more pronounced where particulate fractions from fasted rats were used (A) than with such fractions from refed rats (B). Supernatants from fasted rats (4 animals) raised the synthetase activity in isolated microsomes significantly above that of identical amounts of supernatants from refed rats (4 animals). The relative activation

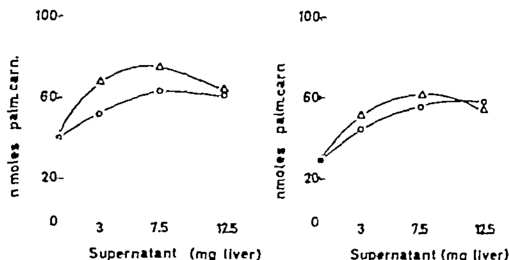


Fig 2. The effect of fasting on he palmityl CoA synthetase activating capacity of particle-free supernatant from ra liver Particulat fraction (5 mg liver) from rats fasted for 24 hrs (left) and from fed rats (right) was preincubated with ATP $MgCl_2$ (both 2.5 mM) nd amounts of supernata t as indicated for 10 minutes. The palmityl-CoA synthetase activity was then ssayed by incubation for 5 min under the conditions given in the legend to Fig 1 The synthetase activity was in all cases corrected for the synthetase contaminating the supernatants.
△—△ supernatan from rats fasted for 24 hrs
○—○ supernatant from rats fed on stock diet.

in the two groups was 72 ± 5 and 58 ± 11 ₀ respectively On the other hand when particulate palmityl-CoA synthetase was incubated with a partially purified activating enzyme the relative activation was less in particles from fasted rat (40 ± 10 than in particles from refed rats (52 ± 8) However this difference was not statisticall significant With large amounts of supernatant the activation was inhibited This might be due to the presence of inactivating factors in the system. A high concentration of protein might also interfere with the formation of palmityl-CoA Farstad 1967 b

Factors influencin palmityl-Co A synthetase inactivation in vitro

The fasting experiments suggested that activation of palmityl-CoA sy thetase occurs i vivo It was therefore of interest to study whether inacti ation of th enzyme also might be invol ed in the regulation of acyl-CoA synthesis.

Fig 3 shows that microsomal palmityl-CoA synthetase was rapidly inacti ated when incubated in 0 1 M Tris buffer pH 7 6) in the presence of 5 mM $MgCl_2$. The inacti ation was markedly reduced in the presence of 2 mM EDTA which complexes endogenous Mg The presence of 5 mM ATP in the incubation med um prevented any nactivation of the added palmityl CoA synthetase Fig 4 show that both NaCl and NaF reduced the nactivation of microsomal synthetase in vitro At all concentrations tested NaF wa more effect ve than NaCl

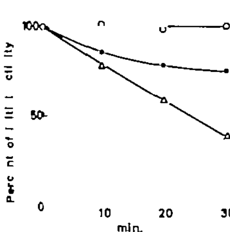


Fig. 3

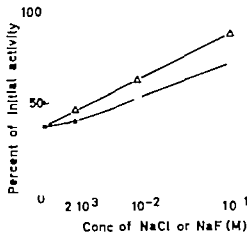


Fig 4

Fig 3. The effect of Mg+ nd ATP on the inactivation of microsomal palmityl-CoA synthetase in vitro.

Identical amounts of isolated microsomes were incuba ed in 0.1 M. Tris buffer (pH 7.6) t 30 ATP EDTA or MgCl was added as indicated.

Samples were taken out at zero time, and after 10, 20 and 30 min. of incubation. Palmityl-CoA synthetase activity was then assayed in all samples as given in the legend to Fig. 1 except that supernatant was omitted.

●—● with 2 mM EDTA
○—○ ith 5 mM ATP
△—△ with 5 mM MgCl

Fig 4. The effect of NaCl nd N F on the inactivation of palmityl-CoA synthetase in vitro

Identical amounts of microsomes were incubated in 0.1 M Tris buffer (pH 7 6) at 30 for 30 min, in the presence of N Cl or NaF as indicated.

Palmityl-CoA synthetase ctivity was then assayed in all tubes as given in the legend to Fig 1 except that supernatant was omitted.

●—● with N Cl, concentrations indicated t the bscissa
△—△ with NaF concentrations indicated t the abscissa

Table II shows an experiment where microsomal palmityl-CoA synthetase was incubated in 0 1 M Tris buffer (pH 7 6) for 30 min at 30° in the presence of different cations. While $MgCl_2$ enhanced the inactivation and CaCl and NaCl reduced the inactivation, KCl had no obvious effect.

Reactivation f partially inactivated palmityl-CoA synthetase by incubation with supernatant. Fig 3 illustrates the results f n experiment in which arious amounts of partially inactivated microsomal palmityl-CoA synthetase were preincubated with a constant amount of a "partially purified acti ating enzyme in the presence of ATP and $MgCl_2$.

The relative increase in the formation of palmityl-carnitine was considerably higher when partially nactivated synthetase was incubated with supernatant than when fresh microsomes were used. The relative ncrease was about 350 and 200 per cent, respectively It is also evident that when starting at the same level of

TABLE II. The effect of different cations on the inactivation of microsomal palmityl-CoA synthetase.

Microsomes were incubated without supernatant in the presence and absence of the stated additions in 0.1 M Tris buffer (pH 7.0) at 30° for 30 min.
The palmityl-CoA synthetase activity was then assayed as given in the legend to Fig. 1 except that supernatant was omitted.

Addition	Conc. mM	Per cent of initial synthetase activity
None	—	58
$MgCl_2$	5	43
$CaCl_2$	5	80
NaCl	50	92
KCl	50	53

palmityl CoA synthetase activity in the absence of supernatant enzyme, the net increase in the formation of palmitylcarnitine as a result of supernatant activation was larger with partially inactivated microsomes than with fresh microsomes. This has been found in several experiments, and makes it probable that reactivation of an inactivated palmityl CoA synthetase really takes place in the presence of supernatant activating enzyme and ATP.

The presence of 10^{-} M cyclic AMP did not increase the supernatant activation of particulate palmityl CoA synthetase either at pH 7.0 or pH 7.6. However, the supernatant preparation used was relatively active, as it increased the activity of palmityl CoA synthetase about 100 per cent even without preincubation.

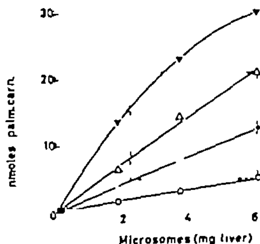


Fig. 5. The effect of supernatant on partially inactivated palmityl-CoA synthetase.

Various amounts of fresh microsomes and partially inactivated microsomes were preincubated for 10 min in 0.1 M Tris buffer, 2.5 mM ATP and 2.5 mM $MgCl_2$ in the presence and absence of a constant amount of partially purified activating enzyme giving optimal activation of palmityl-CoA synthetase.

The synthetase activity was then assayed by incubation for 5 min under the conditions given in the legend to Fig. 1.

○—○ Partially inactivated microsomes in the absence of activating enzyme.
△—△ The same in the presence of activating enzyme.
●—● Fresh microsomes in the absence of activating enzyme.
▼—▼ The same in the presence of activating enzyme.

Discussion

The existence of a palmityl-CoA synthetase activating factor in the particle-free supernatant from several organs in rodents is now established. There are several reasons to assume that the stimulating effect of supernatant on the particle-bound palmityl-CoA synthetase is due to an enzymatic transformation of an inactive or less active synthetase into a more active one

1 The activity of particulate palmityl-CoA synthetase is increased by preincubation with supernatant, ATP and Mg^{++} (Farstad 1967 a)

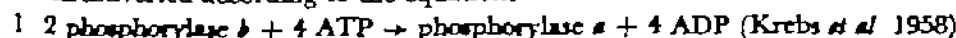
2 The protein exerting the activation can be separated from the palmityl-CoA synthetase contaminating the supernatants (Farstad 1967 a)

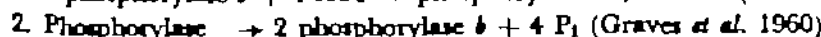
3 The demonstration of two possible sites for ATP participation in the activated system (Farstad 1967 b)

4 The inhibiting effect of excess ATP or Mg^{++} on the palmityl CoA synthetase activating factor is similar to the effect on phosphorylase *b* kinase (Farstad 1967 a Krebs and Fischer 1956, Krebs *et al* 1966)

The present results demonstrate that under optimal conditions, the activation of particulate palmityl-CoA synthetase is proportional with the amount of supernatant activating factor in the medium. Evidence is also presented for an *in vivo* regulation of palmityl-CoA synthetase activity This activity is increased in fasting animals as is also the activity of the activating factor of particle free supernatant.

Previously phosphorylation and dephosphorylation have been demonstrated in the regulation of the activity of some glycogen metabolizing enzymes. Glycogen synthetase is reversibly interconverted between the phosphorylated D form and the dephosphorylated I form (Friedman and Larner 1963) Two different enzymes, a specific kinase and a specific phosphatase are probably involved in these processes (Friedman and Larner 1963) The two forms of glycogen phosphorylase, *a* and *b* are interconverted according to the equations


1 2 phosphorylase *b* + 4 ATP → phosphorylase *a* + 4 ADP (Krebs *et al* 1958)


2 Phosphorylase → 2 phosphorylase *b* + 4 P_i (Graves *et al.* 1960)

Some evidence for a phosphorylation of a mitochondrial acyl CoA synthetase has been reported (Harel *et al* 1961) This phosphorylation was assumed to be dependent on the oxidation of a Krebs cycle intermediate (malate) (Harel *et al* 1961) However the phosphorylation was ATP-dependent, and the possibility exists that the activation was due to contamination with small amounts of the activating factor which is present in particle-free supernatants (Farstad 1967 a)

The present results suggest that the inactivation of palmityl-CoA synthetase in *vitro* at least in part, may be due to *the effect of a phosphoprotein phosphatase.* The complete prevention of inactivation in the presence of ATP suggests that the active form of the synthetase may be phosphorylated The effects of NaCl and NaF are similar to those obtained on a partially purified "phosphorylase rupturing enzyme" by Keller and Cori (1955) The effect of Mg^{++} is in acc

with the observation that a phosphorylase *b* kinase phosphatase is magnesium dependent (Krebs *et al.* 1966). However Keller and Cori (1955) did not obtain any effect of divalent cations on their enzyme. ATP'ases may be involved in the inactivation of palmityl-CoA synthetase *in vitro* by destruction of endogenous ATP. The prevention of inactivation in the absence of exogenous ATP by $CaCl_2$, but not by KCl is similar to observations on microsomal ATP'ases by Skou (1957). The lack of effect of cyclic 3' 5' AMP in our experiments does not exclude the participation of this nucleotide in the activation of the supernatant activating enzyme. Krebs *et al.* (1966) have shown that cyclic 3' 5' AMP increases only the rate of activation, and not the final level of activity of phosphorylase *b* kinase. Thus the activity of the supernatant enzyme in our system might be sufficient to give maximum activation of palmityl CoA synthetase even in the absence of cyclic 3' 5' AMP.

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References

Bremer, J. and K. R. Norum, The mechanism of substrate inhibition of palmityl-coenzyme A: carnitine palmityltransferase by palmityl coenzyme A. *J. biol. Chem.* 1967. *242.* 1744—1748.

De Duve, C., B. C. Pressman, R. Gianetto, R. Wattiaux and F. Appelmans, Tissue fractionation Studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* 1955. *60.* 604—617.

Farstad, M., J. Bremer and K. R. Norum, Long-chain acyl-CoA synthetase in rat liver. A new assay procedure for the enzyme and studies on its intracellular localization. *Biochim. biophys. Acta* (Amst.) 1967. *132.* 492—502.

Farstad, M., A palmityl-CoA synthetase stimulating factor of particle-free supernatants. *Biochim. biophys. Acta* (Amst.) 1967 a. *146.* 272—283.

Farstad, M., Stimulation of the membrane bound palmityl-CoA synthetase by a soluble cytoplasmatic factor — possible regulation mechanism for the enzyme. In: H. Peeters (Ed.) *Protides of the biological Fluids* 1967 b. *15.* 191—196.

Friedman, D. L. and J. Larner, Studies on UDPG-α-glucan transglucosylase. III. Interconversion of two forms of muscle UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. *Biochemistry* 1963. *2.* 669—675.

Graves, D. J., E. D. Fischer and E. G. Krebs, Specificity studies on muscle phosphorylase phosphatase. *J. biol. Chem.* 1960. *235.* 805—809.

Harel, L., E. Mendoza and E. Le Breton, Sur l'existence dans les mitochondries hépatiques de deux formes de thiokinase. Passage de la forme inactive à la forme active. In: P. Desnuelle (Ed.) *The enzymes of lipid metabolism* pp. 60—68, Pergamon Press, London 1960.

Keller, P. J. and G. T. Cori, Purification and properties of the phosphorylase-rupturing enzyme. *J. biol. Chem.* 1955. *214.* 127—134.

Krebs, E. G. and E. H. Fischer, The phosphorylase *b* to *a* converting enzyme of rabbit skeletal muscle. *Biochim. Biophys. Acta.* (Amst.) 1956. *20.* 150—157.

Krebs, E. G., D. S. Love, G. E. Bratvold, K. A. Trayser, W. L. Meyer and E. D. Fischer, Purification and properties of rabbit skeletal muscle phosphorylase *b* kinase. *Biochemistry* 1964. *3.* 1022—1033.

Krebs, E. G., R. J. DeLange, R. G. Kemp and W. D. Riley, Activation of skeletal muscle phosphorylase. *Pharmacol. Rev.* 1966. *18.* 163—171.

Krebs, E. G., A. B. Kent and E. D. Fischer, The muscle phosphorylase *b* kinase reaction. *J. biol. Chem.* 1958. *231.* 73—83.

Norum, K. R., Palmityl-CoA: carnitine palmityltransferase. Purification from calf-liver mitochondria and some properties of the enzyme. *Biochim. Biophys. Acta* (Amst.) 1964. *89.* 95—108.

Skou, J. C., The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim. Biophys. Acta.* (Amst.) 1957. *23.* 394—401.

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The Effect of Some Neutral Macromolecules on the Pattern of Hypotonic Hemolysis

By

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Abstract

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Three neutral polymers, ficoll, dextran and polyethyleneglycol, all inhibit hemoglobin liberation in hypotonic electrolyte solutions. If red cells are suspended in a hypotonic solution which, in the absence of polymer would hemolyse all the cells, only a part of the hemoglobin is liberated. The polymer does not alter the fragility but only the amount of hemoglobin liberated from the individual cells. The inhibitory effect is proportional to the weight concentration of the polymer and independent of the mol. wt. within wide limits. The inhibitor molecules must presumably have a critical minimal size since the trisaccharide raffinose did not inhibit hemoglobin liberation under similar conditions. From the data on the lowest molecular weight dextran fraction it was concluded that the lower limit for dextran was below a molecular weight of about 1700. Complete inhibition of hemoglobin liberation was never obtained and from the evidence available it is suggested that the first 20 per cent, at least, of the hemoglobin may escape with a bulk outflow. The colloid osmotic relationships of the cell during and after hemolysis are discussed and it is concluded that it cannot be decided whether the polymer effect is colloid osmotic in nature.

The hemoglobin loss in human red cells suspended in hypotonic solutions is greatly reduced by the presence of macromolecules such as albumin (Marsden 1954, Lowenstein 1960) or dextran (Marsden, Zade-Oppen and Johansson 1957). It was further shown in the latter paper that the cellular pattern of hemolysis was altered.

In dilute cell suspensions in hypotonic polymer free solutions, all or nearly all individual cells lose none or (nearly) all of their hemoglobin (Hb), i.e. an All-or-None response, and the fraction of Hb liberated from the population is roughly the same as the fraction of red cells transformed into ghosts (Saslow 1929, Parpart 1931, Wilbrandt 1943, Hendry 1947, Marsden, Zade-Oppen and Johansson 1957, Gerhardt 1960).

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With dextran the cellular response may be far from All-or None and the meaning for example of 60 per cent hemolysis in such a system is that all or most of the cells have lost on an a erage 60 per cent or thereabouts of their Hb rather than that 60 per cent of the cells have lost all, or nearly all their Hb.

The aim of this paper is to give more detailed information concerning the effects of three neutral macromolecules dextran, ficoll and polyethyleneglycol on the pat tern of osmotic fragility in human red cells.

Materials

Dextra and the polysucrose ficoll were kindly provided by Pharmacia, Uppsala Sweden, and polyethylene glycol, Carbowax® (PEG) by Union Carbide Europa S.A. U less otherwise stated the polymer fractions sed had the following haracteristics Dextran 250® $\bar{M}$ (umber average mol. wt.) = 1.23 10⁵ $\bar{M}$ (weight average mol. wt.) = 2.5 10⁵ Ficoll® $\bar{M}$ = 4 10 and PEG $\bar{M}$ = 6 – 7.5 10³ Heparin, 125 IU per mg, was kindly provided by Vitrum, Stock holm, Sweden. All other reagent were of highest grade of purity commercially available Blood was taken by enipuncture immediat ly prior t the experiment and wa collected i t bes containing small amount of heparin. The cells were washed three times in one or t olumes of solution containing 125 mM N Cl 5 mM KCl and 25 mM eronal acetat buffer (Michaelis 1931) pH 7.4 (sotonic saline solution) This solution was rega ded as isotonic with blood plasma In the hypotonic sol tions only the NaCl concentration was alt red. The cell wer entrifuged at 3000×g for 20 min and the supernatant emoved by suction.

Methods

P oced

It is easier t pipette accurately packed cells than suspension where the hematoc it is lower sinc n the latter case sedimentation tends to produce errors in the number of cells deliv ed. Therefore one olume of packed cells delivered from mic ometer operated precision syringe was first diluted with about one olume of isotonic saline solution nd the tube was then gent ly shaken t ensure that the cells were aerated nd evenly suspended. Hypotonic solution w then rapidly added from a yringe with fixed endpoints of the piston movement the jection ra from the yringe being sufficient to ensure adequate mixing The relativ of me added aried in different experimen between 36 and 176 as indicated the tables nd figures where the cell dil tion is given After llowing certai time (never less tha 30 min for completion of hemolysis, cells nd media were separated for the analysis of Hb liberated into the medium Except n tw ases the separation was made by entrifugation (3000 g 20 min) fter first having added large 10 or 20 times the olume of the hypotonic suspension) olume of isotonic saline solution I the experimen involving microabsorptiometry the sus pensions were centrif ged 37 500 g without previou dilution I one case filtration was used for the separ tion in parallel w th centrif gation fter dilution. The hypotonic suspen sions were filtered throu h fil ers onsisting of mixed esters of ellulose w th mean por size of 0.45 μ Millipore Corporation Bedford M ss A pressure of about 5 cm of water as used.

For photomic graphy blood or washed nd packed red ell were added directly t the hypotonic media S pensions were sealed with aseline between glass slides and co lips and then photographed in phase ontr t Ca l Zeiss West Germany Hypotonic hemolysis was also followed by cinémicrograph Red ells suspended in an isotonic saline solution were moun ed between slides and cover lips and sealed with seline long two pa allel sides A hypotonic sol tion was added to ne free edge of the coversl p. As the hypotonic sol ion flowed through the preparation hemol sis rred

M eme f hemolysis time by ph se st mic sc py

The duration of hemolysis of individual ells w me sured b hronometer d g he screening of inefilms taken in phase ontrast t peed of 4 frames per sec and jected at 16 frames per sec Hemolysis is usuall first pparent by slight movement of the ll wh h is immedia ly followed by isible hange on ast This movement was aken the starting point The end point is however more diff lt to establish t ery diff l t to state when the on ast ha ge ceases The problem is howev much simplified if he film run backwards sinc now the erminal phase is om ri d an an initial cont as hange

which is much easier to see. The duration of hemolysis as estimated by this method corresponds to the time required for nearly complete Hb liberation because of the sensitivity of the phase contrast microscope for detecting very small optical path differences (Zade-Oppen and Marsden 1968).

Microspectrophotometry

The following procedure was used to determine the amounts of Hb in individual cells.

The red cells were washed once in isotonic saline solution. The Hb in them was then transformed into methemoglobin cyanide (MHbCN) by adding 100 ml of solution containing 8 g/l $NaNO_2$ and 0.208 g/l KCN dissolved in isotonic saline solution to 1 ml of packed cells. The suspension was gently mixed every 5 min during 1/2 hr. The cells were then centrifuged and washed once more.

A cell suspension was then made by mixing equal volumes of packed cells and washing solution. This suspension was kept well mixed in mixing apparatus (Enghoff 1937 p 97) and samples were taken from it for Hb determination, for cell counting, for making hypotonic suspensions containing dextran and for making smears.

The Hb concentration in the cell suspension was determined after making quadruplicate dilutions (1:200) with 1 g/l $NaHCO_3$. This bicarbonate solution was chosen to obtain complete Hb liberation without the disadvantages of more alkaline solutions (Harboe 1959) often used for this purpose.

The cell concentration in the cell suspension was determined with particle-counter (Celloscope, AB Lars Ljungberg, Stockholm, Sweden) after dilution of quadruplicate samples (1:80,000) with isotonic saline solution.

Smears were made from the cell suspension after dilution with equal volumes of the original plasma.

Hemolytic suspensions were made by adding 80 µl cell suspension to 5 ml of solutions containing 0.015 M NaCl, 0.025 M veronal-acetate buffer pH 7.4 and different concentrations of dextran 250.

100 per cent Hb controls were made on each of the hemolytic suspensions, each sample being diluted with an equal volume of 1 g/l $NaHCO_3$. A small amount of digitonin solution (about 3 g/l) was added to complete hemolysis, as the dilution with bicarbonate was not enough for this effect, and the hemolysate was centrifuged to obtain clear supernatant.

Smears were made without further dilution from the hemolytic suspensions.

Finally part of the hemolytic suspension was centrifuged at $37,500 \times g$ (40 min) at 20 °C in an angle-head refrigerated centrifuge. The supernatant was diluted with an equal volume 1 g/l $NaHCO_3$ and the liberated Hb was determined. The results were expressed in per cent of the total Hb in the hemolytic systems.

All smears were dried at room temperature (20—25 °C). They were then immersed in methanol for 2—3 min and again air dried.

The smears were mounted with silicone fluid MS 704 (Midland Silicones Ltd., London, England) and measured on microspectrophotometer (UMSP I Carl Zeiss, West-Germany) at 414 nm using the automatic scanning-integrating system with measuring area diameter of 0.5 µ and line separations also 0.5 µ. The luminous field diaphragm was 5 µ in diameter. The Soret peak of MHbCN is at 420 nm and freshly mounted cells had this value but there was then drift over several hrs to stable value of 414 nm. This value was therefore used for measurements of the integrated absorbances. The silicone fluid 704 was chosen as the mountant both because its refractive index was very near to that of the dried cells, which reduced scattering losses, and because it was found that the cells gave constant values when measured repeatedly in this medium, at least after the peak had stabilized at 414 nm.

A test of the reproducibility of the method was made on methanol fixed HbO_2 cells embedded in glycerol. 11 measurements made over period of two days on one cell gave coefficient of variation of ±4.1 per cent. It was discovered, however after these measurements were made that glycerol was not suitable mountant because there was initially decrease in Hb values with time presumably due to dissolution of some Hb to the glycerol. Since the serial absorbance measurements used did not appear show an downward trend (the probability that 6 measurements on the first day differed from 5 measurements on the second day was low $0.05 > P > 0.02$) the value of 4.1 per cent can be regarded as representative. Similar reproducibility can be expected on cells mounted in MS 704 since reproducibility of the instrument should not depend on the method of sample preparation.

Hb determination in cell suspensions and supernatant

Hb was determined as the MHbCN derivative. The conversion was made by adding 0.1 ml of solution containing 8 g $NaNO_2$, 0.208 g KCN and 4 g $NaHCO_3$ per 100 ml to 4 ml of sample (Zade-Oppen 1960). The absorbance was determined on Beckman DU or Zeiss

PMQ II spectrophotometer at 540 nm or for samples with low Hb concentration, at 420 nm (Zade-Oppen 1960)

100 per cent standa d were obtained by hemolysing completely cell suspensions either by dilution with distilled water or with a 1 100 dilution of isooctylphenoxypolyethoxyethanol (Cutscum® Fisher Scientific Co., Fair Lawn, N J) These hemolysates were not centrif ged.

Results

The determination of liberated Hb

At higher polymer concentrations the density of the solution is similar to, and may exceed that of some of the hemolysed cells. Under these conditions centrifugation gives an incomplete separation of extracellular fluid and cells and an overestimate of the liberated Hb, an error which increases with increasing polymer concentration (Fig 4)

To overcome this two methods were tried with ficoll In the first, the suspension was first diluted with salt solution before centrifugation. This has two results, the cells shrink and become denser and the polymer is diluted, red cing the external density Centrifugal separation is now more efficient. The second method was to filter the hemolytic suspension through a tight (0 45 μ) filter at a low driving pressure ($\sim$5 cm H_2O) Higher pressures produced increased hemolysis which was presumably due to mechanical damage to cells forced into the pores. Because of this limitation, filtration rates are low and become prohibiti ely so with concentrated dextran solutions of high viscosity

The theoretical disadvantage of first diluting and altering the polymer and salt concentrations before separation of cells and supernatant is that this itself may alter the Hb liberation. This is not apparently the case since both methods ga e essentially the same result (see Fig.) This was therefore the method adopted in most of the experiments

The o motı f agility curve and d xt an

Osmotic fragility curves were made with two different concentrations of dextran 250 A shown in Fig 1 Hb liberation was inhibited, each dextran concentration

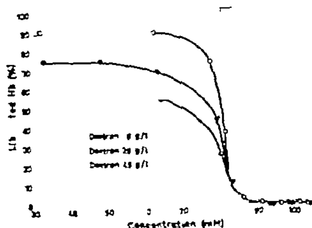


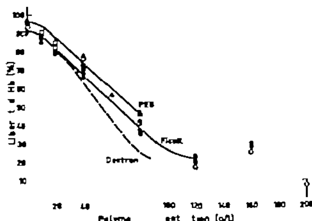
Fig 1 Hb liberation in sol tions containing varying lectrolyte concentrations t two dextran concentrations. Cell dilution 1 61

Fig. _. Maximal Hb liberation as function of concentration for three polymers.

Ficoll. Hb liberation was determined with two techniques of separating cells and medium by filtration through filters with 0.45 μ pore diameter (open circles) and by centrifugation after dilution (1:21) of the suspension with isotonic saline (filled circles). Electrolyte concentration 31 mM. Cell dilution in the hypotonic medium 1 180.

Polyethylene glycol. Hb determination after dilution (1 11) and centrifugation. Electrolyte concentration 32 mM. Cell dilution in the hypotonic medium 1 61

Dextran (dashed line) from Hjelm, Östling and Persson (1966)



being associated with a new maximal level, which varied in an inverse manner with the dextran concentration. Each curve started to rise at about the same salt concentration value and the maximal levels for the three curves were also reached at about the same salt concentration, *i.e.* the salt concentration range in which variations of liberated Hb occurred was about the same for each of the curves.

Inhibitio of Hb liberatio as a function of the polymer concentratio

Fig ? shows the relationship between the Hb liberation and polymer concentration for dextran 250 ficoll and PEG t an external electrolyte concentration of 30 mM, which corresponds to the lowest point of Fig 1 *i.e* a concentration t which the Hb liberation had reached a maximal level.

If the dextran curve is pl tted from the results of Fig. 1 and from measurements on individual cells (Fig 4) th graph obtained fits very well with that given by *Hjelm, Östling and Persson (1966) therefore this latter graph is given in the figure* because t extends over a larger concentration range.

The ficoll curve was obtained with two d fferent methods of separating cells and media, *i.e.* by centrif gation after dil tion w th isotonic medium nd by filtration (see above)

It can be seen that bove co ncentration of about 120 g/l ficoll the nhibitory effect becomes maximal Reproducible alues for Hb liberation were not obtained in dextran concentrations in this range presumably due to the ery high iscosity of the sol tions which made it difficult to achie rapid mixing which seems important for obtaining reproducible results It was however pparent that complete inhibition of Hb liberation was never obta ned.

TABLE I. Liberation of hemoglobin with different molecular weight dextrans and raffinose

Dextran	$\bar{M}$	$\bar{M}$	Nonelectrolyte mOsm	Values of lib. Hb per cent individual	mean
"3"	2 400	3,400	27	51.2 45.0	48.1
"9"	5,900	9 600	12	52.8 55.6	54.2
10 D	6,200	9 400	12	49.6 50.4	50.0
20 D	17 000	22 700	6	54.0 54.6	54.3
2000 D	~ 500,000	~ 2,000,000	2	55.2 54.4	54.8
Raffinose (30.5 g/l)	Mol.wt. = 504		60*	100 per cent faint gray ghosts	

Dextran values calculated from the equation of Hint (1964).
* The osmotic coefficient of raffinose has been assumed to be unity.
Polymer concentrations were 58.5 g/l. Electrolyte concentration 4 mM. Cell dilution 1:38.

The relationship for all these polymers appeared to be approximately linear over a considerable range (ficoll from 10 to about 100 g/l, for dextran up to about 80 g/l and for PEG also up to the maximal concentration used (80 g/l)). It is doubtful, however, whether the differences between the three polymers (Fig. 2) are significant.

Effect of molecular weight of dextran

Table I shows that for dextran fractions at a concentration of 58.5 g/l ranging in $\bar{M}$ between $3.4 \cdot 10^3$ and $2 \cdot 10^6$ the effect on Hb liberation were not significantly different. Raffinose does not have, however, any inhibitory effect. This experiment was made with an electrolyte concentration of 4 mM and in the absence of dextran *the Hb liberation would have been virtually complete. The osmolality of the dextran* solutions was calculated from the equation of Hint (1964). It should be noted that the total osmolality even with the lowest mol. wt. dextran is in the plateau region of the curves in Fig. 1.

Since the viscosities of dextran solutions vary greatly with mol. wt. it is evident that viscosity has no significant effect on Hb liberation. Limiting viscosity numbers for dextran "3" and 2000 are 0.033 and 0.70, respectively.

Duration of hemolysis and stability of final state

The hemolysis of cells in hypotonic solutions was studied under the phase contrast microscope and recorded by cinemicrographs.

TABLE II. Libera ion of hemoglobin after varying equilibration times with dextran 250 t m different concentrations and 30 mM electrolyte. Cell dilution 1 60.

Time	Liberated Hb (per cent) 56.5 g/l dextran individual	mean	91.6 g/l dextran individual	mean
10 min	58.4 56.9	57 7	30.6 32.1	31 4
20	53.8 54 1	53.9	29 7 30.3	30.0
30	53.2 52.9	53.0	31.5 29 7	30 6
1 hr	51 6 56.6	54 1	30.0 29 7	29.8
3	51.3 52.2	51.8	29 4 28.7	29 1
25	53.8 5_.6	53.2	32.1 30.3	31.2
48	— 54 7	54 7	29 7 29 4	29.5

The phase contrast hemolysis times (see M thods) of indi idual cells appeared to be independent of the presence of dextran and ranged between 10 and 25 sec.

Measurements of Hb l'beration in suspensions were done between 30 min and a few hrs after introducing cells into the hypotonic medium.

To exclude therefore the possibility that there is a slow leakage of Hb from the cells over long periods the Hb liberated from a cell suspension was determined after different times in the hypotonic environment. As is shown in Table II the values are stable between 20 min and 48 hrs.

Th pattern of h molysis with n the population

a) Appearances of ells in positive phas ontrast

The microscopic appearance of cell suspension in which hypotonic hemolysis has occurred in the presence or absence of polymers is widely different

Fig 3 a shows the appearance of the cells in a hypotonic polymer free sol tion, in this case about 25 per cent of th cells have been converted into ghosts and bout 25 per cent of the *total Hb* has been *liberated. Fig. 3 b illustrates what happens* when a polymer is present. About 65 per cent of the total Hb has been liberated but t is not possible to classify th cells, which exhibit great diversity of appearance into two distinct groups. Few if any of the cells ha e the appearance of those in the hypotonic dextran free suspension of Fig 3 a. The wid range in the appearance of the phase contrast image of the cells does not necessaril reflect correspond ngl large nations in the actual cells because the image depends on both the thickness of and Hb concentration in the cell The cells of Fig 3 b show much variation in shape (and thickness) Further even f all the cells were regula spheres of equal



Fig 3 The phase contrast appearance of cell hypotonic sol tions.
) 85 mM sal sol tion. Some cells have hemolysed and ppea as faint gray ghost other lls, presumably unhemolysed, appear bright and have prominent halo.
) 25 mM salt containing 38.4 g/l dextran 250. This tonicity in the bsence of dextran would en complet hemolysis. All cells has altered appearance compared with unhemolysed lls and contrast ranging between that of the ghosts and cells in)

olume small ariations in residual Hb alues within the range of about 50 to 75 per cent (n Fig 3 b the mean residual Hb is 35 per cent) will gi e fairly large dif ferences n image contrast (Zade-Oppen and Marsden 1968) In fact in Fig 3 b it can be seen that the population straddles the contrast reversal point, some cells being lighter and the majority darker than the background

b) Microab ption try of ndi d al lls

Fig 4 shows the results of an experiment in which Hb liberation was determined a) from the supernatant Hb concentration in a centrif ged suspension and b) from the mean integrated absorbance alues of either 10 or 20 cells. Hb liberation is ex pressed as residual Hb 100 minus th percent ge liberated and integrated absorbances are expressed relative to th mean value for unhemolysed cells. The cellular Hb content could not be calc lated absolutely nce the extinction coefficient of dried and methanol fixed MHbCN is not known (also the Soret peak shifted in a few hours from an initial al e of 420 nm to a stabl valu of 414 nm) For unhemolysed cells the variation about the mean wa ± 25.0 per cent (± 2 standard deviations n = 20) a value which grees well wit the data of Bahr and Zeitler

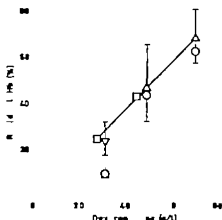


Fig. 4 Minimal residual Hb determined by two methods as function of dextran concentration in hypotonic hemolysis.

The squares are values from Fig. 1 The other points are from one experiment, where cells were diluted (1 125) with solution containing dextran 250 ($\overline{M}$ ~139,000 $\overline{M}$ — 267,000) and 40 mM electrolytes. The circles are the values of 100 minus the Hb in supernatant (after centrifugation with 37,500×g, 40 min) expressed in per cent of total Hb. Triangles are the relative mean integrated absorbance values obtained from measurements of individual cells. Vertical bars show the range The inverted triangle (31.5 g/l dextran) represents selected cells and the interrupted vertical bar represents the range of the cells which were excluded from the data (see results) The numbers of individual cells measured at the different dextran concentration were 10 at 31.5 g/l, 20 at 49 6 g/l and 10 at 70.3 g/L

The continuous straight line which is drawn arbitrarily between four collinear points, is in fact itself collinear with the dextran line in Fig. 2

(1962) From Hb determination and cell counts of the cell suspensions the mean corpuscular Hb was 30.5 pg and the lowest and the highest values for unhemolysed cells calculated by assuming this mean value were 25 pg and 37 pg respectively Statistical data about the distributions of the hemolysed cells are not given because the number of cells measured in some of the groups were too small the ranges, however are shown in Fig 4 The cells hemolysed in 31.5 g/l dextran appeared to consist of two populations with mean values of 23 4 and 12.3 per cent, while the average residual Hb as calculated from the Hb liberated in the suspension was lower than expected (Fig. 4) It seemed therefore most probable that some additional hemolysis had occurred for an unknown reason in this sample The relative mean integrated absorbance value (23 4 per cent) for one of the populations fitted quite well, however to the line of Fig 4 It therefore seems likely that the unexpected high hemolysis obtained in the suspension was due to an effect on only a part of the cell population and that the cells with the higher Hb content represent the population derived from hemolysis in dextran. The weighted mean of the relative mean integrated absorbances of the two cell populations should correspond to the residual Hb value (9 4 per cent) This was not checked, however since it would have involved measuring a considerable number of cells in order to ensure the two populations were correctly represented Measurements were made on five cell from each class.

Provided only the selected cells are included at the lowest dextran concentration all the microabsorptiometric values fit well with the values obtained from measurements on suspensions. The integrated absorbance data indicates that there is a rather large scatter in individual values However the average values for residual Hb, independently of whether they were obtained from individual cells or suspensions, are quite well in accord. In this experiment the Hb liberation in the sus

sions was determined after direct centrifugation which tends to give a somewhat incomplete separation of cells and medium. This may be the reason for the residual Hb value at the highest dextran concentration being somewhat lower than the value derived from absorbance measurements.

It may be asked how it is possible to have a residual Hb (suspension) value lower than either of the mean absorbance figures for the two cell classes. Statistically however there is no significant difference between the Hb liberated and the lower Hb containing cell group. Further no attempt was made to estimate the relative proportions of the two classes of cells. Also cells of the lower Hb group were very faint and difficult to see even at 414 nm so it is also likely that there was a bias in their selection favouring cells with higher Hb content.

Discussion

Measurement of residual Hb or Hb liberation

The main technical problem in the determination of Hb liberation in hypotonic polymer solutions is the separation of cells and extracellular fluid. Filtration of the hemolysate through fine filters proved impossibly slow with dextran sol tions of high viscosity as only a very low filtration pressure could be used without causing additional hemolysis. Fortunately dilution of the ficoll hemolysate with isotonic salt solution, a procedure which gives conditions much more favorable for centrifugal separation, yielded values not significantly different from measurements on filtrates. As the mean values calculated from the absorption measurements on individual cells also give similar values, the correctness of all three methods seems verified.

It may be noted here that in the experiments involving microabsorptiometry the cells were treated with nitrite and cyanide before suspension in hypotonic dextran solutions without affecting th results

Inh bition of Hb libe ation hypotoni olutions by macromolecules

It is known that albumin (Marsden 1954 Lowenstein 1960) and dextran (Marsden and Zade-Oppen 1956, Marsden, Zade-Oppen and Johansson 1957) inhibit Hb liberation from hypotonic solutions These observations were extended here ding also two other neutral polymers, ficoll and PEG Neutral polymers have been studied rather than monodisperse proteins, because in the first place the polymers c be obtained in a wide range of molecular sizes and secondly they are neutral which eliminates solute charge effects and the problem of buffering which occurs at high concentrations.

Th effect of dextran

It is evident (Fig. 1) that while dextran red ices th maximal Hb loss, the external osmolar concentration range between which Hb l beration changes from zero to maximal is very similar to that in a dextran-free solution. The family of curves of Fig 1 would result if dextran reduced only the Hb loss during hemolysis of the ndividual cells without changing the external t nicity at which each cell actually

hemolysis, *i.e.* if dextran did not affect the fragility but only the magnitude of the cellular response. Microscopic observations and measurements support this hypothesis.

a) *Appearances of cells in positive phase c ntrast*

Unhemolysed cells appear in bright contrast while ghosts prod ced by hemolysis in hypotonic polymer free solutions are pale gray At external electrolyte concen trations where hemolysis is incomplete a mixture of these two cell types is seen (Fig. 3 a) Further the fraction of the total Hb liberated is approximately (within about 5 per cent) the same as the fraction of ghosts present (Zade-Oppen and Marsden 1968)

In the presence of dextran, cells in a suspension with the maximal level of liberated Hb present great diversity of appearance (Fig 3 b) which does not, how ever necessarily mean a very wide scatter of individual corpuscular Hb alues, as the phase contrast image is very much dependent on the cell size and thickness. It is thus impossibl to classify these cells into two distinct groups. However in the concentration range where hypotonic hemolysis in dextran-free sol tions is incomplete, it is sometimes possible to distinguish a number of bright cells among a population of otherwise diverse appearance such as in Fig 3 b. In this case the bright cells are presumably unhemolysed and the rest hemolysed cells which have lost only part of their hemoglobin.

b) *Micros terferometry*

Marsden, Zade-Oppen and Johansson (1957) found that cells hemolysed hypotonically in the presence of dextran had a mean dry mass intermediate between that of unhemolysed cells and hypotonic or lytic (photodynamic) ghosts. Further the great majority at least, of cells hemolysed n dextran ppeared to belong to a single population.

The alues gi en, however by these authors for the mean dry mass al es of the cells hemolysed in dextran are not q ite correct because they assumed that the dry mass of *the cell consisted essentially only of Hb* This is, however incorrect, since some of the dextran fraction they used also enters the cells during hemolysis (Marsden and Östling 1959) The possible magnitude of the error can be calculated from the data in the abov two papers for cells hemolysed in 70 g/l dextran the actual mean corpuscular Hb would be bout 45 per cent instead of th value given, *i.e.* 53 per cent. Compared w th Fig 2 the inhibition of Hb liber tion is less, but the significance of this ma be regarded as doubtful

c) *Mi croabsorptiom try*

The number of cells measured was rather small but they re pparently representative since the relative mean integrated absorbances t the different dextran concentrations fit rather well w th residual Hb al es calculated from the Hb liberated from suspensions It is not likely that such good f t would ha e been obtained

from such a small number of cells had there been two cell populations, i.e. hemolysed and unhemolysed, a conclusion which is supported by the range of values at the two higher dextran concentrations.

The case of the lowest dextran concentration where paradoxically there were two cell classes is rather instructive. It seems probable that the excess hemolysis observed was due to the accidental secondary hemolysis of a fraction of the population resulting in two classes of cells in which the absorbance values of the one were about twice the other since the relative mean integrated absorbance of the denser cells agreed well with the expected value. As mentioned previously the relative mean integrated absorbance of the less dense cells which was actually higher than the residual Hb value (suspension) was probably due to sampling errors.

The meaning of the terms "degree of hemolysis" or "fragility"

Provided, as in hypotonic polymer free solutions, the hemolytic process in the individual cells approximates to an All-or-None response then the degree of hemolysis can refer without significant ambiguity to either the fractional number of cells hemolysed (*"complete or fractional number hemolysis"*) or the fraction of some component (e.g. Hb) liberated (*"complete or fractional mass hemolysis"*) (Zade-Oppen and Marsden 1968). In the case of hemolysis in the presence of a polymer this clearly is not the case and hemolysis or fragility must be specified more precisely. Although Hb has been widely used as an index of hemolysis, other lower molecular weight solutes (e.g. reduced glutathione) might be more useful, especially in hypotonic polymer solution (Hjelm, Östling and Persson 1966).

The lower critical molecular size for inhibition of Hb liberation

When red cells were suspended in 60 mM raffinose (+4 mM electrolyte) i.e. an external concentration sufficiently low to produce complete number hemolysis, liberation of Hb was also nearly complete as judged from the phase contrast appearance of the cells. The smallest molecular dextran fraction "3" ($\bar{M}_n$ 400, $\bar{M}_w$ = 3400) on the other hand inhibited Hb liberation apparently just as effectively as did all the other fractions. The Lansing Kraemer (LK) distribution (Lansing and Kraemer 1935) gives a reasonable approximation of the probable mol. wt. distribution (Williams and Saunders 1954; Granath 1958). The LK distribution yields somewhat uncertain values at the lower molecular end and neglecting the lowest 10 per cent of the fraction, 90 per cent of the molecules lie above a mol. wt. of about 1300 while 80 per cent lie above a mol. wt. of 1 00. We can thus conclude that if dextran fraction "3" is equally effective as the other higher mol. wt. fractions that the lower effective molecular size is not larger than an isomaltose with 8 glucose residues (mol. wt. 1315). If this fraction is not completely effective it is unlikely that the ineffective species comprised more than about 10 per cent of the total weight of the fraction; this would raise the lower effective molecular size to that of an eleven glucose residue oligosaccharide (mol. wt. 180?

Since raffinose does not inhibit Hb liberation the critical molecular size th... lies

between that of the triose and the eleven glucose containing oligosaccharide. It must be pointed out, however that this critical range may not be quite the same for molecular species other than the isoamylose (dextran) series.

The maximal inhibition of Hb liberation

It is evident from Fig. 2 that the residual Hb was never greater than about 80 per cent in hypotonic ficoll solutions in which complete number hemolysis occurred.

With dextran the minimal Hb liberation was also probably about 20 per cent. Values in this concentration range are not shown in Fig. 2 because the scatter was rather large.

Ostling (1968) found that in cells hemolysed in hypotonic dextran sol tions there was no dextran uptake if the Hb liberation was less than about 20 per cent for a number of different dextran fractions varying in $\overline{M}_w$ from 10 000 upwards. At higher Hb liberation values dextran was taken up and for any particular fraction the dextran content of the hemolysed cells was approximately a linear function of the Hb liberation minus 20 per cent. Thus, in the case of an Hb liberation of about 20 per cent, escape of Hb occurs without any concomitant inflow of polymer molecules even if the latter are considerably smaller than Hb.

Such a unidirectional permeation would occur if Hb liberation during the initial stages of hypotonic hemolysis were associated with an outward bulk flow of water which prevented diffusional entry of the external dextran, i.e. initially inward dif fusion would be hindered by a counter stream. If such an outward bulk flow oc curred it might continue after the first 20 per cent of the Hb had escaped but at a rate *sufficiently low so as not to prevent the entry of dextran.*

If hypotonic hemolysis results from an osmotically induced overpressure inside th cell which causes the membrane to leak, then any bulk outward flow should be greater at the beginning of hemolysis when presumably the internal pressure is highest. There are some other observations which fit with this concept. Davies, Marsden and Zade-Oppen (1968) reported from cinémicrography of individual cells that the early phase f hemolysis n sometimes ssociated with the expulsion of jets or puffs of Hb These jets are evidence of bulk flow since recoil of the cell may occur on the emission of a jet and a second djacent cell in th path of a jet may be shifted by it. These jets occurred only in the initial stages of hemolysis nd had disappeared within 0 sec

The above observations were made lytic hemolysis where th osmotic gradients are less, but Kochen (1962) als reported jets hypotonic systems. Further See man (1967) and Baker (1967) claimed from lectron microscopic observations that large holes occu transiently during hypotonic hemolysis. H les of this size (up to 300 Å) might in ol bulk flow if there was a hydrostatic pressure difference across the cell membrane

If during hemoly is, the first 20 per cent f the Hb l ves the cell in a bulk ou t flow then either the ll ol me must be reduced r w ter may be ble t enter concomitantly through pores wh h ar to small f even the mallest polymer

TABLE III Calculated osmotic pressures (mOsm) of extracellula polymer (g l) and excess mean residual Hb for t levels of Hb liberation.

Excess residual Hb (per cent)	74		14	
	g l	mOsm	g l	mOsm
Hb		4 1		0..
Dextran "5" ($\bar{S}t$ = 2 4 10⁴)	90	44	20	8.7 (1.0
Dextran 70 ($\bar{S}t_a$ = 3 7 10⁴)	90	10	20	1 4 (0.60)
Dextran ($\bar{S}t$ = 10⁴)	90	3 7	20	0 43
Dextran 2000 ($\bar{S}t$ = 10⁶)	90	3.0	20	0..3
Ficoll	105	3 7	22	0..7

Figures in parentheses are the net osmotic pressures after entry of these dextra fractions int the cell (Östling 1968)

The following data were used Mean corpuscula Hb content 29.9 pg (Bahr and Zeitler 196.) Water content of red cells 0 717 ml ml cells (Savitz, Sidel nd Solomon 1964) Osmotic coefficients of Hb calculated from M Conaghey and Maizels (1961) and mol. wt. of Hb taken as 66,700. Normal ell olume (V_0) 87 μ^3 (Altman and Dittmer 1961) Posthemolytic olume was assumed to be equal to the mean hemolytic cell olume = 1 65 V (Hoffman et al. 1958) The dextran osmolalities were calculated from the equation gi en by Hint (1964) and partial pecific ol me of dextran of 0 61 (Granath 19.8 The ficoll values wer estimated graphically from data kindly provided by Dr K. Granath (Pharmacia, Uppsala, Sweden)

molecules. If such a olume decrease occurred it would not be easy to observe since it might be of very transient existence and for a spherical cell originally μ m iameter t would onl mean a reduction of 0 43 μ in diameter This is not much greater than the uncertainty due to the esolution limit of the light microsc ope and further the refractive index relationships at the cell edge will be altered due mainly to the outflow of Hb thus ncrea ing the optical uncertainties.

O motic lat onsh p betw en nt ac llula Hb and ext ac llular polyme

Table III is an ttempt to calculate an osmotic balance sheet for the cond tions when hemol is is c mpleted In the absence of a polymer the mean residual Hb in the experiments of Fig ? was 6 per cent. The Hb inhibited from escaping by the polymer is then a umed to be the observed residual value minus 6 per cent Thus with 90 per cent and 80 per cent liberation the polymer is assumed t ha pre vented the los of 14 per cent and 4 per cent of the Hb, respect ely

Although 6 per ent of the original Hb is excluded from the calculations it is not implied that it lacks osmotic activity th aim here is merely to estimat the osmolality of the exces Hb that the pol mer causes to be retained Extracellular Hb can be neglected as the ell dilution was rather high

Although the inhibition of Hb liberation does not apparently depend on the mol. wt. of the dextran, influx of dextran oc urs during hemoly is if th mol wt. is below about 10⁵ and is greater the lower the mol wt and the greater the Hb liberation (Östling 1968)

Table III shows osmolality values for four dextran fractions. With an excess residual Hb of 74 per cent (Hb liberation ≈ 20 per cent) it will be recalled that dextran apparently did not enter the cells so that for all fractions the external concentration is the concentration difference between inside and outside. With only 14 per cent excess residual Hb (80 per cent liberation) the influx of the lowest mol. wt. fraction is sufficient to reduce the concentration difference to about one ninth of the external concentration. The influx of dextran 20 is however only sufficient to reduce the osmolality by about a half The ficoll fraction is presumably nonpenetrant Values for PEG have not been included as neither the osmotic coefficient nor the influx were known.

Lowenstein (1960) concluded that the inhibition of hypotonic hemolysis by albumin was a colloid osmotic effect. But she did not apparently take into account the possibility that albumin penetrated into the cell as was found by Klibansky De Vries and Katchalsky (1960) As can be seen in Table III the calculated excess residual Hb osmolalities are less than the polymer values (even using the reduced values for the penetrant dextran) except in the case of 14 per cent excess residual Hb for the two higher mol. wt. dextrans. Th absolute differences between the latter and the Hb value are small and their significance, if any is impossible to assess.

The osmolality values, calculated from the external polymer concentrations (Table III) thus do not apply during the course of hemolysis since the membrane is not impermeable to these solutes and the relationship of the real osmolality (π_r) at an instant during hemolysis to that (π_c) calculated from the concentration will be

$$\pi_r = \sigma \pi_c$$

where σ is the reflection coefficient (Staverman 1951) which may vary between zero for solutes which permeate as easily as the solvent to unity for solutes to which the membrane is entirely impermeable. Since the porosity of the membrane varies in an unknown way during hemolysis and since the reflection coefficients of the polymer depend on the molecular size the osmotic relationships during hemolysis must be exceedingly complex.

The calculated osmolalities of dextran fractions which re nonpenetrant represent, however a constant effective value ($\sigma = 1$) For the penetrant fractions th effective osmotic pressures will depend on th concentration difference cross the membrane and the magnitude of the reflection coefficient which will decrease with decreasing molecular size. The inhibitory effect of dextran appears to be only weight concentration dependent and ndependent of the mol. wt

If w suppose that different dextran fractions of equal weight concentration have equal inhibitory effects because of equal effective colloid osmotic pressures we are in fact stating that at any particular concentration

$$\sigma(\pi^o - \pi^i_c) = \text{constant}$$

where the superscripts o and refer to the extracellular and intracellular compartments respectively A conseq en e of this would be that for all fractions above

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Formation of Extracellular Adenosine Triphosphate by Human Erythrocytes

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Abstract

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The formation of extracellular adenosine triphosphate by human erythrocytes was studied. The red cells were incubated with (^{32}P)-orthophosphate, together with all necessary substrates and cofactors of the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions (*complete system*) and also with an *incomplete system* lacking the phosphorylated substrates and cofactors. Finally, the red cells were incubated with the complete system lacking only one phosphorylated cofactor. All incubations were performed under isotonic conditions.

At 1 min incubation, three times more $AT^{32}P$ was formed by the red cells incubated with the complete system than with those incubated with the incomplete system. All the resulting $AT^{32}P$ formed by the added phosphorylated substrates and cofactors present in the complete system could be recovered in the extracellular medium. Only an insignificant part of this $AT^{32}P$ was probably formed by enzymes which were eluted out into the surrounding medium. It is therefore reasonable to infer that most of the $AT^{32}P$ present in the extracellular medium was formed by the enzymes located on the surface of the red cells. No $AT^{32}P$ was found in the extracellular medium of red cells incubated with the incomplete system, nor with the complete system lacking only one phosphorylated cofactor. Thus, none of the intracellular ADP, NAD or glyceraldehyde-3-phosphoric acid could be utilized in ATP formation on the red cell surface.

Isolated membraneous structures of human red cells are capable of forming ATP when incubated with substrates and cofactors of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD oxidoreductase (phosphorylating) E.C. 1.2.1.12) and phosphoglycerate kinase (ATP: D-3-phosphoglycerate 1-phosphotransferase E.C. 2.7.2.3) (Ronquist and Ågren 1966, Parker and Hoffman 1967, Ronquist 1967, Schrier 1967). Green *et al.* (1965) concluded, after experiments with a preparation of beef erythrocyte membranes, that the complete glycolytic enzyme complex is associated with the plasma membrane and is not free in solution in the intact cell. It was therefore of interest to ascertain whether there is an extracellular formation of ATP by intact human erythrocytes incubated with the aforementioned substrates and cofactors.

In the present investigation, intact erythrocytes were incubated with the substrates

and cofactors (including $^{32}P_i$) of glyceraldehyde-3-phosphate dehydrogenase and of phosphoglycerate kinase. The intra- and extracellular distribution of the labelled products was studied after incubation under various conditions. All incubations were performed in an isotonic medium (295—298 milliosmols). The possibility that intracellular enzymes, substrates and cofactors could have diffused out of the red cells and taken part in extracellular reactions had to be taken into account.

However, results given in the present communication indicate that only a minor part of the extracellular AT^{32}P can have been formed by enzymes in the surrounding medium. Evidence is presented that most of the extracellular AT^{32}P was formed by enzymes located on the surface of the red cells.

Material and Methods

Synthesis of labeled ATP

(γ-^{32}P) ATP was prepared according to Engström (1962).

Analytical methods

Radioactivity and orthophosphate were determined as described in a previous paper (Ronquist 1967). Hemoglobin was determined by the cyanmethemoglobin method (Crosby and Houchin 1958). Osmolarity was measured in an Advanced Osmometer, Model 64—31 (Advanced Instruments, Inc., Massachusetts). Hematocrit was determined in an Adams readacrit centrifuge, ES-7331-4-62 (Clay-Adams, New York 10, N.Y.).

Special chemicals

ATP (sodium salt), ADP (sodium salt), GSH (crystalline), 3-GAP and NAD were obtained from Sigma Chemical Company, St. Louis, M. U.S.A.

Preparation of red cells

Venous blood samples from healthy blood donors were collected in heparinized tubes at the Blood Transfusion Service, University Hospital, Uppsala (by courtesy of Dr C. F. Högman). The blood was centrifuged at 650 × g for 20 min, and the plasma and the buffy coat layer were removed. The red cells were washed 3 times with 3 volumes of 0.15 *M* sodium chloride. When red cells were used suspended in 0.15 *M* NaCl containing 6 (w/v) Dextran (Dextran T 40, Mw = 41 800, M_n = 25,700, η = 0.210, AB Pharmacia, Uppsala, Sweden) the third washing was performed with this solution. All preparative steps were carried out at 4 C. The red cells were used immediately after preparation.

Incubation of red cells

The hematocrit of the suspension of isolated red cells was adjusted to 38—40 with 0.15 M sodium chloride or 0.15 M sodium chloride containing 6 Dextran. 19.5 ml of this suspension was prewarmed and incubated at 37 C for various times with the following substances: Tris-acetic acid buffer pH 7.5, 300 µmoles; NaCl, 125 µmoles; $MgCl_2$, 25 µmoles; GSH, 5.0 µmoles; 3-GAP 3.5 µmoles; ^{32}P 2.0 µmoles; NAD 5.0 µmoles; ADP 7.0 µmoles in a final volume of 20 ml. The ^{32}P was purified before use as previously described (Ronquist 1967). An incubation medium containing all these substances is denoted as the "*complete system*" in contrast to the "*incomplete system*" containing only the Tris buffer, NaCl, $MgCl_2$, GSH and the ^{32}P. Finally, in some experiments, the red cells were incubated with different amounts of ADP and ATP or (^{32}P) ATP together with the Tris buffer, NaCl, $MgCl_2$ and GSH.

The incubations were carried out during gentle stirring in polyethylene tubes. In the experiments accounted for in Table I and Fig. 3 incubation was stopped by addition of 3.0 ml of 3 N perchloric acid followed by 10 ml of ice-cold distilled water. The mixture was then centrifuged, and the clear acid supernatant filtered before neutralization with 1 M potassium hydroxide to about pH 6.8—7 (indicator paper).

Abbreviations: ^{32}P = radioactive orthophosphate, ATP = adenosine triphosphate, ADP = adenosine diphosphate, NAD = nicotinamide adenine dinucleotide, oxidized form, 3-GAP = glyceraldehyde-3-phosphoric acid, 2,3 DPGA = 2,3 diphosphoglyceric acid, GSH = glutathione, reduced form ... moglobin

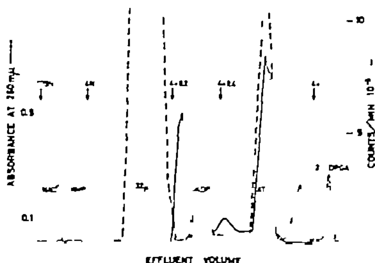


Fig. 1. Dowex 1 X2 (format[e]) [c]hromatogram of the external medi[u]m (supernatant) from [a] red cell suspension incubated for 1 min with the [c]omplet[e] system (see text). The supernatant obtained after centrifugation of the incubated red [ce]ll suspension was [a]pplied t[o a] Dowex 1 X2 column, as described in Methods. Elution was performed stepwise as follows: 0.75 N formic acid; 4 N formic acid 0.2 M [a]mmonium format[e] 4 N formic acid; 0.4 M ammonium format[e] -4 N formic acid; 1 M ammonium format[e] -4 N formic acid.

I[n] all other experiments, the samples were immediately centrifuged [at] 800 × g for 14 min after the incubation period. This implies [t]hat the incubation times given in T[a]bles II, III and IV are not directl[y] comparable with [t]hose in Table I [a]nd Fig. 3. The [c]lear faintly pink [s]upernatant was gently deca[nt]ed int[o a t]ube containing 1.5 ml of 3 N perchloric acid. The [r]esidue [c]onsisting of the packed red cells, was mixed once with 10 ml of cold (4° C) 0.15 M sodium chlorid[e or] 0.15 M sodium [c]hloride [c]ontaining 6[%] Dextran, and [c]entrifuged as just described. The precipitation procedure was also carried out on this supernatant. The two supernatants were combined [a]nd neutralized with 1 M potass[iu]m hydroxide to pH 6.8—7 (indicator paper). Separa[te an]alyses showed that 88—92[%] of all the extracellula[r] AT[32]P obtained was present in the first supernatant. The packed red [c]ells were then trea[t]ed w[i]th 3 ml of 3 M perchloric acid and diluted with 10 ml of ice-cold w[at]er. The mixture was [c]entrifuged [a]nd the [c]lear supernatant fil[t]ered. The precipitat[e] was washed once w[i]th ic[e]-cold distilled [w]ater, after which the combined supernatants were neutralized with 1 M potassium hydroxide [to a]bou[t] pH 6.8—7.0 (indicator paper).

To ascertain whether any sol[u]ble enzymes were present in the extracellular medium, 20 ml of prewarmed (37° C) [u]nsu[p]ple[me]nted red [c]ell suspension (hematocrit 38—40[%]) w[as c]entrifuged at 800 × g for 14 min. The [c]lear supernatant containing either isotonic sodium [c]hlorid[e] solution or isotonic sodium [c]hloride with 6[%] Dextran [w]as incubated with the components of [t]he *complete system* in [a] final [v]olume of 10 ml. This was the *blank*. The reaction was stopped by adding 1.5 ml of 3 N perchloric acid, followed by neutralization to pH 6.8—7 with 1 M potassium hydroxide.

Isolation and identification of [32]*P-labelled* [*co*]*mp*[*o*]*unds*

The neutralized samples were stored for [at] lea[st] 8 hours at 4° C bef[o]re fil[t]ra[t]ion [a]nd Dowex 1 ion-exchange [c]hroma[t]ography. The [c]hromatographic procedure wa[s t]he same as that described earlier (Ronquist 1967) except tha[t] elut[i]on was started w[it]h 0.7[5] N formic acid t[o] separat[e] NAD and AMP (Fig. 1).

Further isolation and identifica[ti]on of [t]he [32]P-labelled compounds (orthophospha[te], AMP, ADP, ATP [a]nd 2,3 DPGA) were perf[o]rmed as previously described (Ronquist 1967).

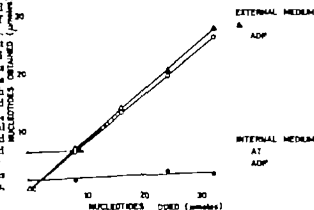


Fig. 2. Recovery of ADP and ATP in the external (supernatant) and internal medium after addition of the nucleotides to intact red cells. Red cell suspensions (hematocrit 30—40) were incubated for 1 min at 37° C with various amounts of nucleotides (ADP and ATP) as given in the figure, together with 300 µmoles of Tris-acetic acid buffer pH 7.5, 25 µmoles of $MgCl_2$ and 5 0 µmoles of GSH in final volume of 20 ml. The incubated suspensions were centrifuged, and the nucleotide content of the external and internal medium was determined as described in Methods.

Results

Penetration of nucleotides into the red cells

From the data shown in Fig 2 evidence was obtained that the predominating part of the nucleotides added to the intact erythrocytes could be recovered in the external medium.

In two similar experiments, the red cells were incubated for 1 min with 0.5 µmoles of AT³²P followed by centrifugation as described in Methods, to separate the extra and intracellular phases. Altogether 95 and 96 %, respectively of the total radioactivity was found in the external medium. It therefore seems reasonable to infer that the penetration of external ADP and ATP into the red cell is of negligible magnitude.

Labelling of ATP with ³²Pᵢ in a cell suspension incubated with the complete and incomplete systems

Fig 3 shows that there is a significant difference already at 10 sec between the total amount of AT³²P formed by the two systems. A short lag period is seen in the curve of the incomplete system. Such a lag in the labelling of ATP was reported in a previous study (Tatibana *et al* 1960) in which erythrocytes were incubated for short periods with Pᵢ only On the other hand on incubating the red cells in the complete system, the incorporation of Pᵢ into ATP increased linearly with time without the initial lag period On explanation of these findings could be the existence of glycolytic enzymes on the red cell surface. It was therefore necessary to exclude the possibility of any diffusion of intracellular enzymes into the external medium.

Red cells incubated with the complete and incomplete systems in the presence and absence of Dextran T40

Hjelm *et al* (1966) found that a relation exists between the Dextran concentration and the magnitude of the disappearance of molecules from the red cells suspend

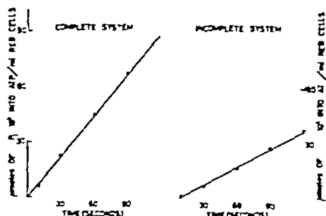


Fig. 3. Time course of appearance of ^{32}P in ATP. Red cell suspensions (haematocrit 35—40 %) were incubated with the complete and incomplete systems (see text) for short periods at 37° C in a final volume of 20 ml. Reactions were terminated by the addition of 5 ml of 3 N perchloric acid. The ATP was separated and analyzed as described in the text.

a haemolytic hypotonic salt solution. They showed e. g. that the escape of Hb or hexokinase from the red cells suspended in a hypotonic solution could be inhibited to about 50 % by the presence of 6 % Dextran in the medium.

In accordance with this observation, an addition of Dextran to our system would probably have inhibited a leakage of intracellular enzymes to the extracellular phase. Intact red cells were therefore incubated with the complete or incomplete systems in the presence or absence of 6 % Dextran.

No definite difference was present between the cell suspensions containing 6 % Dextran and those lacking it. A difference could have been expected if the enzymes had leaked out of the red cells. (See Table I, II and III.)

Incorporation of ^{32}P-orthophosphate into extracellular and intracellular $AT^{32}P$

Further evidence of an extracellular production of ATP was obtained by the results given in Table II and III, which show that all the "additional" ATP formed (over and above that formed in the incomplete system) — which can be attributed to the added phosphorylated substrates and cofactors in the complete system — was in the external medium. The labelling of ADP may have been due to the presence of adenylate kinase (ATP: AMP phosphotransferase, E.C. 2.7.4.3) inside the cell, as well as on the cell surface. If ATP had been synthesized only in the glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions, it should have had the same specific radioactivity as the added $^{32}P_i$. However, the specific radioactivity of the ATP formed was lower, indicating an additional way of synthesis of extracellular ATP. This additional extracellular ATP formation may be accounted for by the adenylate kinase activity on the cell surface. Further support to the surmise that adenylate kinase may be present on the cell surface was the observation that AMP was formed in about stoichiometric amounts to ATP on incubation with a system containing ADP. The presence of adenylate kinase on the red cell membrane is in agreement with an earlier investigation by Kashket and Denstedt (1958).

TABLE I Total AD³²P and AT³²P formation on incubation with complete and incomplete systems. 19.5 ml of red cell suspension (hematocrit 40 %) in either 0.15 M sodium chloride or sodium chloride containing 6 % Dextran T 40 were incubated with the complete and incomplete systems (see text) for various times at 37 °C. The red cells were precipitated with perchloric acid immediately after the incubation period. The incorporation of $^{32}P_i$ into ADP, ATP and 2,3 DPGA is expressed in µmoles of orthophosphate × 10^6 per ml of red cells. The total amounts of ADP, ATP and 2,3 DPGA are expressed in µmoles per ml of red cells. Bracketed figures denote results obtained from experiments with the red cells suspended in the isotonic sodium chloride containing 6 % Dextran. For further details, see text.

Complete system	ADP (^{32}P incorporation)	ADP (total amount)	ATP (^{32}P incorporation)	ATP (total amount)	2,3 DPGA (^{32}P incorporation)	2,3 DPGA (total amount)
1 min	12.7 (12.4)	0.58 (0.56)	46.6 (47.8)	1.04 (1.02)	0.7 (0.7)	3.8 (3.8)
5 min	34.8 (34.0)	0.57 (0.56)	128.6 (130.8)	1.02 (1.02)	6.5 (6.1)	3.8 (3.8)
10 min	59.0 (63.7)	0.58 (0.57)	199.2 (198.3)	1.03 (1.01)	13.7 (14.1)	3.8 (3.7)
15 min	77.6 (89.4)	0.60 (0.58)	283.2 (291.4)	1.03 (1.02)	20.6 (20.0)	3.9 (3.7)
20 min	105.3 (111.2)	0.59 (0.58)	346.2 (359.1)	1.01 (0.99)	33.8 (30.5)	3.8 (3.7)
Incomplete system						
1 min	3.9 (4.1)	0.19 (0.20)	16.2 (16.5)	0.86 (0.83)	0.7 (0.6)	3.9 (3.8)
5 min	8.6 (9.8)	0.19 (0.21)	36.4 (39.6)	0.84 (0.83)	6.5 (6.3)	3.8 (3.9)
10 min	23.2 (24.9)	0.21 (0.18)	119.2 (122.3)	0.85 (0.84)	13.3 (12.8)	3.8 (3.7)
15 min	34.9 (36.2)	0.21 (0.21)	185.0 (185.1)	0.85 (0.85)	20.4 (21.1)	3.9 (3.7)
20 min	34.2 (50.7)	0.20 (0.21)	241.5 (248.4)	0.83 (0.84)	34.0 (34.8)	3.8 (3.8)

Table II also shows that the incorporation of $^{32}P_i$ into ADP and ATP by the blank was very small compared to that by a red cell suspension. The blank did, however, have adenylate kinase activity. The ADP converted into ATP by the blank was of the same order of magnitude as that of the extracellular medium of red cells incubated with the complete system. This activity of the blank may possibly have been eluted during the prewarming at 37 °C in sodium chloride or sodium chloride-Dextran solution.

Red cells incubated in the incomplete system did not form any extracellular ATP. Therefore, ADP, NAD and 3-GAP cannot have leaked out of the cells into the extracellular medium. The incorporation of ^{32}P into the intracellular ADP, ATP and 2,3 DPGA was of the same magnitude as that of red cells incubated in the complete system (Table III). The total amounts of ADP in Tables I, II and III indicate that the adenylate kinase reaction on the red cell membrane had reached an equilibrium already at one minute's incubation. This rapid equilibrium should be compared to an earlier observation by Tatibana *et al.* (1958), who found a rapid conversion of ADP to ATP by a haemolysate of human red cells. They incubated 2 ml of a haemolysate (corresponding to 0.5 ml of erythrocytes) with 5 µmoles of ADP at 37 °C, and found that this reaction was complete in about 20 ...

TABLE II The distribution of the labelled nucleotides between the extracellular medi m (super red cell suspension (hematocrit 40) in 0.15 N sodi m chloride (or in 0 15 N sodium t 37 C. The supernatant (blank 9.5 ml) obtained after centrifugation of similar (ala- as th red cell suspension. The incorporation of $^{32}P_i$ int ADP ATP nd 2,3 DPGA b ATP and 2,3 DPGA are expressed in μmoles per ml of red cells. Bracketed figures denot taining 6 Dextran. For further details, see text.

Extracellula medium supernatant	ADP ($^{32}P_i$ incorporation)	ADP (total amount)	ATP ($^{32}P_i$ incorpo-ration)
1 min	3 1 ..6.6	0.35 (0.3()	87 1 (91.2)
5 min	62 7 (62.9)	0.36 (0.36)	102.4 (103.1)
10 min	4.8 6.7	0.34 (0.35)	124.8 (1 1.9)
15 min	100.1 97..	0.3((0.3)	159 1 (158 6)
20 min	12'4 118 7	0 3((0.3()	182.3 (179.3)
Intracellular medium (cell phase)			
1 min	44 6 4(.9)	0.21 (0.22)	220.6 (201 7)
5 min	6 2 80 8	0.21 (0.21	349 6 (322 7)
10 min	87 1 9 5)	0.20 (0.21	437.8 (431.2)
15 min	121 7 1 1.8	0.20 (0.22	552.3 (560.1)
20 mi	143.0 148..	0.22 (0 21	635.3 6.2.0)
Incubated supernatan blank			
1 min	1 0 1	0 33 0.33	1.8 2.2
m	4 1 3 9	0.32 0.33)	6.3 6 8
10 min	5 4 5 5	0 33 0.32	8.. (8
15 min	7 2 7 3	0 33 (0 31	11 5 (11 9
20 min	10 9 11 2	0 32 0 32	17 1 19 0

The specific radioactivity of the intracellular and extracellular orthophosphate changed slightl during the ncubation period The specific radioactivity of the extracellular orthophosphate was 23.8 10^4 cpm μmole mean) at one minute incubation and decreased to 18.1 10^4 cpm μmole mean) at 20 minutes ncubation. The corresponding figures of the ntracellular pecific radioactivity of the orthophosphate were 4 6 10^4 and 6 9 10^4 cpm μmole respectively (means) Thus a slight increase occurred in the pec fic radioactivity of the intracellular orthophosphate These changes in specific radioactivity may be accounted for by the relatively slow influx of P_i into the red cell Equilibrium f the P_i concentration between the extracellular and intracellula media had not been reached within 20 minutes incubation.

Red cells were also incubated n the c mplete system lacking either ADP NAD 3-GAP or $MgCl_2$ (Table IV The ncorporation of P_i into external ATP was

natant) and intracellular medium (cell phase) after incubation in the complete system. 19.5 ml of chloride containing 6 % Dextran T 40) were incubated with the complete system for various times cubated) red cell suspension was incubated in total olume of 10 ml under the same conditions expressed in µmoles of orthophosphat × 10³ per ml of packed red cells. The total amounts of ADP results obtained from experiments with the red cells suspended in the isotonic sodium chloride con-

ATP (total mount)	2,3 DPGA ($^{32}P_i$ incorporation)	2,3 DPGA (total amount)	Hb (of cell phase alue)
0 21 (0.22)	0.1 (0.1)	0 (0)	0.28 (0.16)
0.21 (0.21)	0.1 (0.1)	0 (0)	0.27 (0.15)
0 20 (0.23)	0.2 (0.1)	0 (0)	0.28 (0 15)
0.21 (0.22)	0.2 (0.2)	0 (0)	0.29 (0 16)
0 20 (0.22)	0.2 (0.2)	0 (0)	0.29 (0.16)
0.87 (0.87)	17.2 (15.6)	3.6 (3 7)	100 (100)
0.87 (0 87)	37 4 (34 4)	3.7 (3.7)	100 (100)
0 86 (0 87)	55 6 (56.1)	3 6 (3 6)	100 (100)
0.85 (0.86)	80.4 (84 0)	3.6 (3.6)	100 (100)
0.85 (0.86)	102.1 (100.8)	3 6 (3.7)	100 (100)
0.23 (0.23)	0 (0)	0 (0)	0 26 (0.15)
0.22 (0 23)	0 (0)	0 (0)	0.26 (0 15)
0.22 (0.24)	0 (0)	0 (0)	0.26 (0.15)
0 21 (0.23)	0 (0)	0 (0)	0.26 (0.15)
0 22 (0.22)	0 (0)	0 (0)	0 26 (0.15)

negligible in the first three cases and comparable with that in the incomplete system Thus, none of the intracellular ADP NAD or 3-GAP seems to have participated to any measurable extent in the extracellular reactions. The omission of $MgCl_2$ from the incubation medium had no effect on the $^{32}P_i$ ncorporation into ATP Th intra cellular formation of ^{32}P labelled ATP was high in all cases, and of the same magni tude as that in the complet system, suggesting that the entry of $^{32}P_i$ into the cell was independent of the processes involved in the extracellular production of AT^{32}P

Discussion

A significant d fference was present between the amount f ATP formed by red cell incubated in he complete system nd that i the ncomplet system The
of 6 Dextran T40 in the incubation medi m did not influence the f

TABLE III Distribution of the labelled nucleotides between the extracellular medium (supernatant) ell uspension (hematocrit 40 %) i 0.15 M sodium hloride (or in 0.15 M sodium times t 37° C. The incorporation of $^{32}P_i$ into ADP ATP and 2,3 DPGA is expressed DPGA are expressed in µmoles per ml of red cells. Bracketed figures denot results 6 % Dextran. For further details, see text.

Extracellular medium (supernatant)	ADP ($^{32}P_i$ incorporation)	ADP (total mount)	ATP ($^{32}P_i$ incorporation)
1 min	0 (0	0 (0)	0 (0)
5 min	0 (0)	0 (0)	0 (0)
10 min	0.1 (0.1)	0 (0)	0.1 (0.1)
15 mi	0.1 (0.1)	0 (0)	0.1 (0 1)
20 min	0.1 (0 1)	0 (0)	0.2 (0 1)
Intracellular medium (cell phase)			
1 min	40.4 (44.8)	0.18 (0.18)	218.0 (221 6)
5 min	66.0 (68.6)	0 19 (0 18)	356.4 (359 1)
10 min	79 6 (82.6)	0 18 (0.17)	432.9 (438 8)
15 min	102.3 (112.2)	0.19 (0.19)	540 8 (541 7)
20 min	122.2 (125 4)	0.20 (0 19)	612 1 (648.4)

TABLE IV ADP P and ATP P formation on incubation with the complete and incomplet systems, as w ll with the complete system lacking only one cofactor 19.5 ml of red cell suspension hematocrit 38—40) in 0 15 M sodium chloride were incubated with the complet and incomplet systems, as well as with the complete system lacking either ADP NAD 3-GAP or $MgCl_2$. Incubation time was 1 min t 37° C, followed by the centrifugation procedure as described in Methods. The results are given in µmoles of orthophosphat 10^4 ncorporated into ADP and ATP respectively per ml of red cells Bracketed figures denot he total mount of the nucleotide in µmoles per ml of red ells For further details, see ex

	Extracellular medium				Intracellular medium	
	ADP		ATP		ADP	ATP
Complete	1 8	0 3	89	0 22	42 6 (0 21)	2 6 0 (0.88)
Incomplet	0	0	0	(0	43 4 (0.19)	229 7 (0 8)
Complete minus ADP	0	0	0	(0	46.3 (0.19)	234.3 (0 87)
Complet minus NAD	0	0 3	0	0 21	43 1 (0.20)	221.8 (0 88)
Complet minus 3-GAP	0	0 3	0	0 22)	41.9 (0.20)	224 1 (0 87)
Complet minus $MgCl_2$	47 9	0 37	85 3	0.21	40.3 (0 19)	232.2 (0 87)

and intracellular medium (cell phase) after incubation in the incomplete system. 19.5 ml of red chloride containing 6 % Dextran T 40) were incubated with the incomplete system for arious in µmoles of orthophosphate × 10^{8} per ml of red cells. The total amounts of ADP ATP and 2,3 obtained from experiments with the red cells suspended in the isotonic sodium hloride containing

ATP (total amount)	2,3 DPGA ($^{32}P_i$ incorporation)	2,3 DPGA (total amount)	Hb (% of cell phase value)
0 (0)	0 (0)	0 (0)	0.26 (0.14)
0 (0)	0 (0)	0 (0)	0.26 (0.15)
0 (0)	0 (0)	0 (0)	0.26 (0.15)
0 (0)	0 (0)	0 (0)	0.27 (0 15)
0 (0)	0 (0)	0 (0)	0.27 (0.15)
0.83 (0.83)	16.9 (17.5)	3.6 (3.5)	100 (100)
0.84 (0 83)	38.6 (39 4)	3 6 (3 6)	100 (100)
0 84 (0 84)	59.2 (55.1)	3.6 (3.5)	100 (100)
0 83 (0 82)	78 3 (76.7)	3.5 (3.6)	100 (100)
0 83 (0.83)	104.3 (101 6)	3.6 (3.6)	100 (100)

ATP by the cells. Furthermore, the amounts of $AT^{32}P$ formed by the blanks (sodium chloride or sodium chloride Dextran centrifugate from a red cell suspension) were negligible. It therefore seems reasonable to infer that the extracellular $^{32}P_i$ labelling of ATP observed during incubation of the red cells can scarcely have been caused by any enzyme eluted from the interior of the cell. Thus, these results give strong evidence that the extracellular $AT^{32}P$ had been formed by the enzymes bound to the surface of the red cells.

Adenylate kinase activity was always observed in the blanks. No measurable difference existed between the amount of (^{32}P) ATP formed in the blank containing only sodium chloride solution and that in the blank containing sodium chlorid Dextran solution. Thus, for reasons ment oned above (Hjelm *et al* 1966) it is assumed that this activity was washed out of the cell surface by the sodium chloride or sodium chloride Dextran solution, rather than ha ing leaked out from the cell interior

The present results indicat that extracellular ADP and ATP are not delivered to the cell interior under the conditions used in the present work. *E. g* the amounts of intracellular $AD^{32}P$ and $AT^{32}P$ were bout th same in the complet and incomplet systems These results d not agree with those of Kashket and Denstedt (1958) who claimed that ADP could pass from th outside inwards (but not in the opposit direction) They tried t expla their results by suggesting that ADP after ha ing entered the external part of the membrane, underwent dismutation to ATP and AMP These products could then pass freely into or out of the cell. However

incubation conditions were not the same. Thus the medium of Kashket and Denstedt contained glucose and a 14 times higher concentration of ADP than that in the present investigation. The incubation periods were also longer in the reported work (up to 1 hr).

It is evident from the present investigation (Table IV) that neither ADP, NAD nor 3-GAP inside the cell can be used outside in the reactions on the red cell membrane. Nor can the intracellular ADP act as an acceptor of the reactions on the red cell surface, since no increase in the intracellular formation of AT^{32}P could be detected after incubation with the complete system lacking ADP.

Lindberg (1948, 1950) studied the equilibration of the external inorganic phosphate with both inorganic phosphate and energy-rich phosphate in unfertilized and fertilized eggs of the sea urchin. He concluded that ATP was formed from orthophosphate on the outer surface of the egg. Neither of these phosphate fractions was able to penetrate the membrane of the unfertilized egg. There was, on the other hand, a slow penetration of orthophosphate into the fertilized eggs.

In previous studies of orthophosphate penetration into red cells, it has been suggested that the phosphate may be bound to an intermediary compound when passing the cell membrane (Gourley 1952, Prankerd and Altman 1954, Bartlett 1958, Gerlach, Fleckenstein and Gross 1958, Schrier 1963). Among several hypothetical schemes, it has been proposed that the phosphate enters the cell by formation of ATP on the cell membrane (Gourley 1952). Other workers have suggested that 1,3-diphosphoglyceric acid may be the intermediary step between the external orthophosphate and the internal ATP (Prankerd and Altman 1954, Bartlett 1958, Gerlach, Fleckenstein and Gross 1958, Schrier 1963).

Definite conclusion about the mechanism of the orthophosphate penetration through the red cell membrane cannot be drawn from the results given in this paper.

I wish to express my gratitude to Professor G. Ågren for all his help and interest in this work. I am also indebted to Mr. S. Eklund, Mrs. M. Eriksson and Mr. T. Fransson for their technical assistance.

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References

BARTLETT, G. R., Organization of red cell glycolytic enzymes. Cell coat phosphorus transfer. Ann. N.Y. Acad. Sci. 1958, 75, 110–114.

CHERRY, W. H. and D. N. HOBSON, Preparing standard solutions of [illegible] Blood 1957, 12, 1135–1136.

EKSTRÖM, L., Formation of [illegible] phosphorylserine during hydrolysis of [illegible] P-labelled glucose phosphate by alkaline phosphatase from calf intestinal mucosa and Escherichia coli. [illegible] 1962, 19, 1[illegible]–140.

GERLACH, E., A. FLECKENSTEIN and E. GROSS, Der intermediäre Phosphat-Stoffwechsel des Menschen-Erythrocyten. Papierchromatographische Studien unter Verwendung von ^{32}P-markiertem Orthophosphat. Pflügers Arch. ges. Physiol. 1958, [illegible] 78–[illegible]5.

GOURLEY, D. R. H., The role of adenosine triphosphate in the transport of phosphate in the human erythrocyte. Arch. Biochem. 1952, [illegible]

GRAY, D. E., E. MEYER, H. O. H[illegible], S. H. P[illegible], B. S[illegible], G. P. B[illegible] and H. BERG, Association of [illegible] with the cell membrane [illegible] glycolytic enzymes of the red blood corpuscle and yeast. Arch. Biochem. [illegible] 11 [illegible]3–447.

HJELM, M. S. G. ÖSTLING and A. E. G. PERSSON, The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of Dextran. *Acta physiol. scand.* 1966. *67* 43—49

KASHKET, S. and O. F. DENSTEDT. The metabolism of the erythrocyte. XV. Adenylate kinase of the erythrocyte. *Canad. J. Biochem.* 1958. *36* 1057—1067

LINDBERG, O. On the turnover of adenosine triphosphate in the sea urchin egg. *Arkiv Kemi* 1948. *26 B* 13 1—4

LINDBERG, O. On surface reactions in the sea urchin egg. *Exp. Cell Res.* 1950 *1* 105—114

PARKER, J. C. and J. F. HOFFMAN, The role of membrane phosphoglycerate kinase in the control of glycolytic rate by active cation transport in human red blood cells. *J. gen. Physiol.* 1967 *50* 893—916.

PRANKERD, T. A. J. and K. I. ALTMAN, A study of the metabolism of phosphorus in mammalian red cells. *Biochem. J.* 1954 *58* 622—633.

RONQUIST, G. and G. ÅGREN, Formation of adenosine triphosphate by human erythrocyte ghosts. *Nature* (Lond.) 1966. *209* 1090—1091

RONQUIST, G., Formation of adenosine triphosphate by a membrane fraction from human erythrocytes. *Acta chem. scand.* 1967 *21* 1484—1494.

SCHRIER, S. L., Studies of the metabolism of human erythrocyte membranes. *J. clin. Invest.* 1963 *42* 756—766

SCHRIER, S. L., ATP synthesis in human erythrocyte membranes. *Biochim. Biophys. Acta* (Amst.) 1967 *135* 591—598.

TATIBANA, M., M. NAKAO and H. YOSHIKAWA, Adenylate kinase in human erythrocytes. *J. Biochem.* (Tokyo) 1958. 45. 1037—1038.

TATIBANA, M. K. MIYAMOTO, T. ODAKA and M. NAKAO, Phosphorus metabolism in human erythrocyte. V. Incorporation of ^{32}P during very short time intervals. *J. Biochem.* (Tokyo) 1960. *48* 683—690.

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Renal Excretion of Chloride, Bromide and Thiocyanate during Water Diuresis

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Abstract

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The purpose of the present investigation was to examine the suggestion advanced by Sauerman (1957) that differing mechanisms of renal tubular reabsorption of chloride and bromide might be detected by studying the anion excretion during water diuresis. Simultaneous excretion of Cl and ^{82}Br was therefore studied before and during water diuresis in 10 normal human subjects. The excreted fraction of filtered bromide was invariably 30—35 per cent lower than that of chloride independently of serum bromide concentration (range 0.001—10 meq/litre), excretion percentage of chloride (0.3—4 per cent) and of the rate of urinary production (0.6—14 ml/min). It was concluded that there is no evidence that the renal reabsorption mechanism differentiates between chloride and bromide.

Simultaneous determination of chloride, bromide and thiocyanate excretion confirmed that only the excretion of these anions is increased during water diuresis.

The ultrafiltrability of the three anions was determined and considered when the filtered amounts were calculated. The ratios between anion concentration in ultrafiltrate and serum water were: chloride 1.0, bromide 0.98 and thiocyanate 0.77.

It has been maintained that the fractions of filtered chloride and bromide which are eventually excreted in the urine are identical (F es 1911, Moller 1932). However, there is evidence from animal experiments that the tubular reabsorption of bromide is more complete than the reabsorption of chloride (Walser and Rahill 1966). The present investigation was carried out to examine whether the mechanisms of reabsorption in the normal human kidney distinguish between chloride and bromide. The plan of this study followed the suggestion advanced by Sauerman (1957) that differing mechanisms of tubular reabsorption of anions may unmask themselves during water diuresis.

A comparative study of chloride and bromide excretion during water diuresis has not been performed previously. Therefore, excretion of both anions was examined in 10 individuals with normal renal function. Reabsorption of chloride was found to be less complete than that of bromide, but the ratio between the excreted fractions

of filtered chloride and bromide was not affected to any significant degree by th rate of urinary flow Simultaneous examination of thiocyanate excretion was carried out in two experiments, confirming the observation of Saugman (1957) that the excretion of thiocyanate is considerably increased during water diuresis.

Methods

The excretion of chloride and bromide was examined in 10 male subjects without any history of renal disease. All had protein-free urine and normal creatinine clearance I tw of the experiments (no. 9 and 10) the excretion of thiocyanate was studied as well.

No food or fluid was allowed in period of 12 hours preceding the study 0.1 mC of ^{82}Br-labelled ammonium bromide dissolved in 10 ml of an 0 15 M NaCl solution was injected intravenously 3—4 hrs before the first control period. ^{82}Br-labelled NH_4Br with an initial specific activity of 4 mC per meq bromide was obtained from AEK, Riso, Denmark, and was employed for experiment within one week after delivery

Before the experiments no. 1 and 2 total dose of 120 mmoles of sodium bromide was given by mouth over a period of 2 to 3 days to obtain bromide concentration of 5—10 meq per litr of serum. 40 meq of NaSCN was given intravenously in 0.5 M solution 3 hrs before the experiments no. 9 and 10.

During the experimental periods the subject was placed in the supine position, except when oiding. In the experiments no. 1 2 and 3 urine was collected through an indwelling catheter and complete emptying was ensured by flushing the bladder with air Catheterization was omitted in the remaining experiments without any inadvertent effect on the creatinine clearances, which were determined in every experimental period. After control period of 61—107 min duration, water di resis was induced by an initial intake of 1 litre of tap water followed by 250 ml every 15 min until brisk diuresis had been established. Blood samples for chemical analyses and radioactive countings were drawn through an indwelling enous cannula at the beginning of each collection period and at the end of the study

The following determinations were carried out on serum and urine samples. ^{82}Br was counted in 3 ml samples in γ-well scintillation counter All countings were extended sufficiently to obtain counting error below 1 per cent. Adequate corrections were employed for radioacti decay of ^{82}Br Chemical determination of bromide in rine and serum was carried out in experiments 1 and 2 by the method of Todd et al (1955) However t was found that the colour reaction with gold chloride was not stable when samples of concentrated urine were analysed. The urinary concentration of bromide i these tw experiments was, therefore, calculated from the specific activity of bromide in serum and from the cou ting rate of ^{82}Br in the urine samples. I expts. 3—10 bromide concentrations were calculated from the counting rates of serum and urine and the specific activity of the injected bromide, assuming that no bromide was present in the organism prior to the study Chloride was determined by mercurimetric titration (Brun 1949) corrections being applied for the presence of bromide in experiments 1 and 2 and of thiocyana in experiments 9 and 10 Thiocyanate was determined with F (NO3) as described by Saugman (1957) and creatinine was analysed according to the method of Owen *et al.* (1954) The coefficient of ariation of the analytical methods was estimated from the results of consecutiv analyses performed in duplicate ^{82}Br in serum and urine 0.56 per cent (=100) chloride in serum and concentrated urine 0.91 per cent (=50) chloride in dilute urine (range of concentration 5—25 meq per litre) 1 35 per cent (=16) and thiocyanate in serum and urine 0 71 per ent (=19

The amounts of bromide hloride, and hiocyanate filtered were alculated according to the equation

Anion filtered (μeq/min) = C W A_s A_u/A_w, C being the endogenous reatinine learance (ml/min) W be erage fractional water olume of 10 normal serum samples (0 941) A_s the concentration of the nion in serum water (μeq/ml) and A_u A_w the ratio between the concentrations of he anion in ultrafiltrate and serum water The l rafiltrability of hloride, bromide and thiocyanate in normal serum samples was determined with the technique of Lavietes (1937 10 ml of serum was equilibrated at 38 C and t arbon dioxide partial pressure of 40 mm Hg. During the equilibration period 0 01—0 03 C of ^{82}Br labelled NH_4Br was added in olume of maximally 40 μl. Bromide concentrations between 1 and 10 μeq/l were obtained by adding 100 μl of sodium bromide sol ion of appropriate concentration (expts. 3—10 Table III) The ltrafiltrability of thiocy nate was studied at serum concentrations comparable to those found in the excretion study (— 9 meq per litre Table III) I hese experiments 20—30 μl of 1 0 M solution of NaSCN were added

TABLE I Simul neous renal excretion of chloride and bromide ([82]Br) i 10 h man individuals period before induction of w ter diuresis I the following periods the experimental

Exp. no.	Period no.	Period min	Creatinine clearance ml/min	Urine output ml/ml	Serum chloride meq/l	Chloride meq/min filtered	Chloride meq/min excreted
1	1	61	130	0.68	93.5	12.64	0.088
	2	50	136	2.24	95.8	13.33	0.097
	3	72	143	12.94	92.6	13.47	0.097
2	1	107	105	0.99	102.6	11.02	0.205
	2	32	111	5.01	99.2	11.24	0.154
	3	52	113	11.46	99.5	11.49	0.159
3	1	63	118	0.67	106.8	12.82	0.066
	2	39	120	0.77	105.0	12.82	0.035
	3	24	155	8.21	103.0	16.23	0.082
	4	43	143	14.07	101.5	14.80	0.091
4	1	90	160	0.57	108.3	17.02	0.111
	2	58	151	1.83	108.3	16.70	0.103
	3	36	153	11.78	106.5	16.61	0.118
5	1	89	131	0.58	107.3	14.30	0.163
	2	60	130	0.83	106.5	14.09	0.151
	3	23	120	6.64	101.5	12.74	0.097
	4	29	114	10.97	103.0	12.01	0.096
	1	98	111	2.0	105.5	11.99	0.147
	2	93	159	9.30	101.6	16.49	0.171
7	1	97	136	0.71	104.0	14.44	0.176
	2	48	142	0.96	103.5	14.97	0.183
	3	31	150	6.94	103.5	15.78	0.167
	4	61	118	11.44	102.2	12.51	0.108
8	1	105	131	0.61	108.0	14.46	0.142
	2	4	126	1.21	107.0	13.74	0.145
	3	83	13[illegible]	14.69	106.5	14.73	0.146
9	1	85	1[illegible]1	0.79	106.5	17.31	0.128
	2	90	[illegible]	2.18	101.7	17.61	0.130
	3	40	1[illegible]4	8.50	103.8	17.32	0.101
	4	91	14[illegible]	1.37	103.3	15.23	0.087
	5	97	16[illegible]	[illegible]43	103.3	17.82	0.101
10	1	96	167	1.20	102.6	17.42	0.370
	2	70	161	2.37	99.7	16.39	0.242
	3	71	151	14.4	98.5	15.14	0.151

the serum sample After temperature nd CO_2 equilibration 6 ml of serum were transf rred to the Lavletes pparat Ultrafilt ation was arried out t pressure of 200 mm Hg at 38 C through thin cellophane membrane Cuprophane PT 150) After 2—4 hrs 1 –2 ml of ltrafiltrat could be collected I few experiment ul rafil ration of bromide w con- tinued after the initial sampling The ncrea ng protein concentra ion in the serum con-

before and during w ter diuresis. The first period presented in each experiment is the control data were grouped according to the rate of urinary flow: <1 1—5 5—10, or >10 ml per mi

Serum bromide µeq/l	Bromide µeq/min filtered	Bromide µeq/min excreted	Excretion percentage (1) Chloride	Excretion percentage (2) Bromide	Relative bromide excretion (2)/(1)
7940	1010	5.2	0.70	0.51	0.73
7750	1040	5.7	0.73	0.55	0 75
7420	1040	6.1	0.72	0.59	0 82
6100	630	10.68	2.40	1 70	0 71
5850	636	5.6	1.37	0.88	0.64
5790	643	4.88	1.38	0 76	0.55
6 00	0 693	0 0024	0.51	0.35	0.69
5.95	0.699	0 0012	0.28	0 17	0 61
5.70	0.877	0.0025	0.51	0.29	0.57
5.69	0 799	0.0026	0 62	0.33	0 53
2.26	0.334	0.0016	0.63	0.45	0.71
2.25	0.331	0.0015	0.63	0.45	0 71
2.19	0.329	0.0013	0.71	0.36	0.51
1 18	0 151	0.0011	1 14	0 73	0.64
1.16	0.148	0.0011	1 09	0 74	0.68
1 12	0.131	0 00066	0.76	0.50	0 66
1 11	0 125	0.00066	0 80	0.53	0.66
2.40	0.262	0 0021	1.23	0.80	0.65
2.30	0.359	0 0022	1 04	0.61	0.59
3 92	0.524	0.0040	1.22	0 76	0.62
3.88	0 540	0.0042	1 22	0 78	0.64
3 87	0.561	0 0038	1 06	0 68	0 64
3 78	0 438	0.0024	0 88	0.55	0 63
32..	4 19	0 0284	0.98	0 68	0 69
31 7	3 92	0 0299	1 05	0.71	0.72
30 9	4 11	0 0293	0.99	0 73	0.74
5 09	0 806	0.0043	0.73	0.53	0 73
5 02	0.813	0.0042	0 74	0 52	0.70
4.94	0.794	0.0030	0 .8	0 38	0 66
5.00	0 709	0.0028	0 57	0 41	0 7.
5 03	0 833	0 0033	0.57	0 40	0 70
2.31	0 378	0.0060	2.12	1 59	0 75
2.25	0 356	0.0038	1 48	1 07	0 72
2.17	0 321	0 0023	1.00	0 72	0 72

partment migh be anticipated to cause an increased shif of anions the ltrafiltrate How ever such an effect of continued ultrafil ration could not be observed Similar observations hav been reported repeatedly (Lavietes 1937 Rose 1 57 Salminen 1961)

Chemical analyses and determination of ^{82}Br in serum and ultrafiltrate were c rried out b the methods cited above W ter content of serum was determined after drying serum t constant weight at 105 C.

Results

Data of the excretion of chloride and bromide are listed in Table I The excretion percentage of bromide was lower than that of chloride in every period. The ratio between bromide and chloride excretion percentage was unaffected by variation of serum bromide concentration between 0.001 and 10 meq per litre For the clearness of presentation the results were grouped according to the rate of urinary flow (cf. text of Table I) Each period presented in Table I therefore represents from 1 to 4 experimental periods. The urinary output in 21 control periods before the induction of water diuresis averaged 0.9 ml per minute and the corresponding value during 30 periods of maximal water diuresis was 11 6 ml per minute. The average ratio between the excretion percentages of bromide and chloride in the control periods was 0 69 (SE 0 09 n = 1) This was not significantly different from the average ratio of 0 67 (SE 0 01) found in 47 experimental periods after induction of diuresis. In 6 of the 10 expts. the ratio decreased with increasing urinary flow but this trend was not statistically significant.

The excretion percentages of chloride found during the control periods (range 0.34— 56) reflect the spontaneous range of variation in the rate of chloride excretion. Excretion percentages of chloride which were initially above 1.0 decreased during water diuresis (cf expts. ? 5 6. 7 10) In Table II we have compared the reduction of chloride and bromide excretion in these 5 expts. It appears that reduction of chloride excretion is accompanied by a reduction of bromide excretion of similar magnitude

Chloride bromide and thiocyanate excretion was studied sim ltaneou ly in exp. 9 and 10 confirming the finding of Saugman that the excretion percentage of thiocyanate increases during water diuresis. Fig 1 shows the course of the rinary output and th excretion percentages of the three anions d ring one of these experiments. Excretion f chlorid and bromide was lightly decreased during water di resis wherea th excretion f thiocyanate increased with increasi g urinary flow

Kruhøffer 1950 ha suggested that protein binding of bromide ions might be responsible for err neous determination of the relation between bromide and chloride excretion Th eref re the ultrafiltrability of the three anions was tudied and appropriat corrections pplied when alculating th amounts of chlorid bromide and thiocyanat filtered n the glomeruli The results of th e study of ultrafiltrability are hown in Tabl III In greement with the findings of Saugman (1957) w found that the distribution ratio of hloride between serum water and it ltra filtrat 1 0? is slightl lower than the alue f 1 05 to be expected from a Donnan distribution of chloride between serum water and its ltrafiltrat (Van Sl k 1926) The ultrafiltrability of bromid wa gnific ntly lower than that of chloride the ratio of Br ultrafiltrate/Br serum w ter being 0 98 ($p < 0.005$) The corresponding

TABLE II Comparison of the decrease of the excretion percentages of chloride and bromide. The results were extracted from the fi experiments in which the excretion percentage of chloride during the control periods was abov 1 (viz. T ble I)

Exp. no.	(1) Chloride excretion percentage (control periods)	(2) Chloride excretion percentage (water diuresis)	(2)/(1)	(3) Bromide excretion percentage (control period)	(4) Bromide excretion percentage (water diuresis)	(4)/(3)
2	2.40	1.38	0 58	1 70	0.76	0 45
5	1 14	0 78	0.68	0 73	0.52	0.71
6	1.23	1.04	0.85	0.80	0.61	0.76
7	1.22	0.95	0 78	0 76	0 60	0.79
10	2.12	1.00	0.47	1.59	0.72	0 45

average ratio of 0.77 found for thiocyanate is somewhat lower than the value found at identical serum concentrations by Saugman, but agrees with the results of Scheinberg and Kowalski (1950) and of Davson (1955)

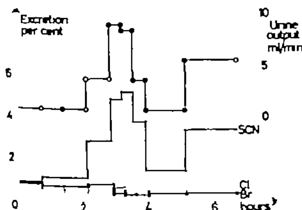


Fig 1 Urine output O—O and excretion percentages of chloride, bromide, and thiocyanate before and during water diuresis. The decrease in urinary flow after three hours was accompanied by severe headache and was presumably due to endoge nous release of antidiuretic hormone. (Exp. 9 T ble I)
Chloride x—x
Bromide O—O
Thiocyanat —

Discussion

S nce a fallacy due to binding of anions to plasma proteins can be excluded the present study it must be concluded that the excretion percentage of bromide in human urine is significantly lower than that of chloride. Endogenous creatin ne clearance determined by the analytical method employed, exceeds inulin clearance by a factor of bout 1.3 so that use of creatinine clearance as n exact measure of glomerular filtration is open to criticism The values of filtered chloride and bromi in Tabl I are accordingly on an average too high, and the excretion percen

TABLE III The ultrafiltrability of serum bromide, thiocyanate and chloride. Ultrafiltration was carried out at 38 °C at CO partial pressure of 40 mm Hg (*cf.* method section). Calculated amounts of filtered anions (Table I) were corrected according to the results of the ultrafiltration study

Anions tested	(1) Chloride meq/l serum water	(2) Bromide or thiocyanate meq/l serum water	(3) Chloride meq/l ultrafiltrate	(4) Bromide or thiocyanate meq/l ultrafiltrate	(3)/(1) Ultrafiltration ratio Cl	(4)/(2) Ultrafiltration ratio Br or SCN
Cl Br	108.8	1.20 × 10⁻	110.0	1.16 × 10⁻	1.011	0.967
Cl Br	112.8	7.59 × 10⁻	116.0	6.98 × 10	1.028	0.920
Cl Br	114.9	2.13	117.9	2.06	1.026	0.967
Cl Br	109.6	4.26	108.7	4.29	0.992	1.007
Cl Br	112.2	6.35	116.4	6.51	1.037	1.025
Cl Br	114.3	6.35	114.6	6.38	1.003	1.005
Cl Br	123.4	8.51	123.6	8.41	1.002	0.988
Cl Br	109.6	8.51	111.8	8.17	1.020	0.960
Cl Br	116.0	10.63	116.5	10.4[illegible]	1.001	0.984
Cl Br	104.0	10.72	111.3	10.72	1.070	1.000
Cl SCN	117.1	1.98	121.3	1.66	1.036	0.838
Cl SCN	107.1	2.40	10[illegible].1	1.88	0.985	0.783
Cl SCN	10[illegible].7	2.55	107.1	1.91	1.013	0.749
Cl SCN	114.0	2.71	114.0	2.02	1.000	0.745
Cl SCN	108.7	2.72	114.1	1.90	1.050	0.699
Cl SCN	108.8	2.87	111.8	2.22	1.028	0.774
				Cl mean	1.019	Br 0.987
				SD	0.022	0.030
						SCN 0.765
						SD 0.04[illegible]

too low. However, the clearance determination is without influence on the parameter of principal interest, the relative bromide excretion, which is presented in the last column of Table I. The excretion percentage of ^{82}Br was approximately 30 per cent lower than that of chloride independently of the rate of urinary flow. Neither was the relation between the excretion of the two anions affected by variations of the chloride excretion percentage between 0.3 and 2.4 or by an increase of serum bromide concentration from 1 to 10 meq per litre (Table I). In the experiments in which a substantial decrease in the fractional excretion of chloride was observed during water diuresis we found an almost identical reduction of the excretion percentages of chloride and bromide (Table II). Our results have clearly established that there is no similarity between the changes of bromide and thiocyanate excretion during water diuresis (Fig. 1).

As mentioned in the introduction some investigators have reported that the ex cretion percentages of chloride and bromide are identical (Frey 1911 Moller 1952) whereas others have found that the fractional excretion of filtered chloride exceeds that of bromide by a factor of 1 4—2.5 (Palmer and Clarke 1933 Bodansky and Modell 1941 Winkler and Smith 1943 Wolf and Eadie 1960 Walser and Rahill 1966) It is worth noting that the experiments of Frey and Moller were car ried out on rabbits and the experiments of the other authors on dogs. It is, therefore, possible that a preferential reabsorption of bromide over chloride aries be tween different mammalian species, being pronounced in the dog but almost absent in the rabbit. In iew of such probable species differences there is a remarkable lack of information about the relative excretion of chloride and bromide in the human kidney Gamble *et al.* (1953) reported an a erage ratio Br (urine/plasma)/Cl (urine/plasma) of 0 76 after the administration of tracer doses of ^{82}Br and of ^{36}Cl to 6 patients. Husar and Holley (1956) stated without further details that the preferential excretion of chloride over bromide by the kidney found in dog experi ments also holds for humans" This statement is confirmed by the present investi gation.

The most detailed study of chloride and bromide excretion has been carried out in dog experiments by Walser and Rahill (1966) They studied the excretion of the anions in saltdepleted and in salt loaded animals. They found an exponential rela tion between the excreted fractions of chloride and bromide

(excreted/filtered bromide) = (excreted/filtered chloride)^k

The average value of k (which they called the discrimination factor) was 1 13 The variation of chloride turn-over in our material was too small to decid whether a similar exponential relation applied to the excretion of chlorid and bromide by th human kidney The results of Tabl I are equally well described by a linea nd by an exponential relation between the excreted fractions of chloride nd bromid To examine whether an exponential relation exists, it will be necessary to carry out excretion studies during salt loading The average alue of th discrimination f ctor k, calculated by inserting th results of Table I into th above equation, was 1 09 (range 1.04—1 14) W th this al e of k the relation between the excreted frac tions of bromide and chloride would be 0 67 t chloride excretion percentage of 1 but 0.87 at a chloride excretion percentage of 20.

Neither a linear nor an exponential relationship between chloride and bromide excretion yields a clue to the problem whether or not active processes are nvolved in the renal reabsorption of chloride (Walser and Rahill 1966) Saugman (1957) concluded that the difference bet een th excretion percentages of chloride and thiocyanate during w ter d uresis ggested that chlorid i reabsorbed by pecific active transport mechanism in the distal tubule, and h proposed that a tudy of chloride and bromid excretion might show that bromide excretion is increased d ring water diuresis, indicating that chlorid is preferred to bromide when nions are reabsorbed against considerable concentration gradient the distal tubule.

The present res lts ha e demonstrated that preferential absorption of chloride

is not unmasked during water diuresis. The fraction of filtered bromide excreted was invariably 30—35 per cent lower than that of chloride (Table I) Also the uniform changes of the excretion percentages (Table II) seem to indicate that the mechanisms regulating the reabsorption of the two anions were identical

Evidence for active reabsorption of chloride in the distal tubule of the rat has been presented by Rector and Clapp (1962) These authors found that the chloride concentration in urine during sulphate diuresis was occasionally lowered below 1 meq/l, which was the lowest concentration of chloride compatible with passive distribution of chloride between tubular fluid and plasma in the presence of a transtubular potential difference of -120 mV (lumen negatively charged) During maximal water diuresis the average concentration of chloride in urine in the present experiments fell from 20.4 meq/l (S.E. 2.5 n = 10) in the control periods to 0.7 meq/l (S.E. 0.9 n = 10) The average chloride concentration in serum water was about 100 meq/l. Assuming that chloride reaches equilibrium between distal tubular fluid and plasma, a transtubular potential of -60 mV during the period of maximal water diuresis is, therefore the lowest potential difference consistent with a passive distribution of chloride between tubular fluid and plasma. It is not known whether a transtubular potential difference of -60 mV or more develops over the distal tubular wall during water diuresis and it is, therefore, not possible to decide whether active reabsorption of chloride and bromide took place in our experiments. If it is assumed that chloride was actively reabsorbed, it is necessary to postulate that this transport mechanism prefers bromide to chloride because the fraction of filtered bromide which was excreted was always lower than that of chloride Moreover it must be a feature of a common transport mechanism to chloride and bromide that it becomes loaded with bromide and chloride in a proportion bearing a constant relation to the ratio between the concentrations in which the two ions are present in the tubular fluid even when the ratio of Cl to Br —as in the present experiments —varies between 10 and 10^3 (Table I)

Therefore we find it most likely that the reabsorptive movements of chloride and bromide in the present experiments were passive The increase of the excretion percentage of thiocyanate accompanying water diuresis is consistent with passive reabsorption of anions if it is assumed that the permeability of the distal tubule to chloride and bromide is high independently of the presence of antidiuretic hormone whereas the permeability towards thiocyanate is lowered significantly in the absence of antidiuretic hormone (Fig. 1

References

Boo sky O and W Moer The differential excretion of bromide nd hloride ions and its role in bromide re en ion *J Pharma ol p Ther* 1941 73 51 64
Br s C., Klorbestemmelse E mikrometode *Nord Med* 1949 4 1774—1776.
D vson H Adsorption of thioc ana be plasma proteins. *Brit J Ophth* 1 19 5 39 681—681
Fary E. v Die Ursache der Bromretention Vergleich der Brom- nd Chlorausscheidung durch die Nieren. *Z p Path Th* 1911 8 29 70

GAMBLE, J. L., J. S. ROBERTSON, CH. A. HANNIGAN, CH. G. FOSTER and L. E. FARR, Chloride, bromide, sodium and sucrose spaces in man. *J. clin. Invest.* 1953. *32* 483—489.
HUSSAR, A. E. and H. L. HOLLEY, Treatment of bromide intoxication with mercurial diuretics. *Amer. J. Med.* 1956. *20* 100—106.
KRUHØFFER, P., In *Studies on water-electrolyte excretion and glomerular activity in the mammalian kidney.* Copenhagen, Rosenkilde & Bagger 1950. pp. 241.
LAVIETES, P. H., Anaerobic ultrafiltration. *J. biol. Chem.* 1937. *120* 267—275.
MØLLER, K. O., Über die Ursache der Bromidretention. *Arch. exp. Pathol. Pharmakol.* 1932. *165* 244—260.
OWEN, J. A., B. IGGO, F. J. SCANDRETT and C. P. STEWART, The determination of creatinine in plasma or serum and in urine. A critical examination. *Biochem. J.* 1954. *58* 426—437.
PALMER, J. W. and H. T. CLARKE, The elimination of bromides from the blood stream. *J. biol. Chem.* 1932. *99* 435—444.
RECTOR, F. C. and J. R. CLAPP, Evidence for active chloride reabsorption in the distal renal tubule of the rat. *J. clin. Invest.* 1962. *41* 101—107.
ROSE, G. A., Determination of the ionised and ultrafilterable calcium of normal human plasma. *Clin. chim. Acta* 1957. *2* 227—245.
S[illegible], S., In *Studies on the ultrafiltrability of serum sodium and potassium.* Helsinki, Kirjapaino oy Savo, 1961. pp. 71.
SA[illegible], B., In *Det diffusible anionrest udskillelse i patologiske tilstande.* København, Nyt Nordisk Forlag. 1937. pp. 169.
SCHEINBERG, I. H. and H. J. KOWALSKI, The binding of thiocyanate to albumin in normal human serum and defibrinated blood with reference to the determination of "thiocyanate space". *J. clin. Invest.* 1950. *29* 475—482.
TODD, J. C., A. H. SANFORD and B. B. WELLS, 12. *Clinical diagnosis by laboratory methods.* Philadelphia and London, Saunders 1953. pp. 460.
VAN SLYKE, D. D., In *Factors affecting the distribution of electrolytes, water and gases in the animal body.* Philadelphia, J. B. Lippincott Company 1926. pp. 62.
WALSER, M. and W. J. RAHILL, Renal tubular reabsorption of bromide compared with chloride. *Clin. Sci.* 1966. *30* 191—205.
WINKLER, A. W. and P. K. SMITH, Renal excretion of potassium salts. *Amer. J. Physiol.* 1942. *137* 94—103.
WOLF, R. L. and EADIE, G. S., Reabsorption of bromide by the kidney. *Amer. J. Physiol.* 1960. *163* 436—441.

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Intracellular Distribution of Lipolytic Enzymes in the Rat Pancreas

By

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Abstract

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Intracellular distribution of lipase (glycerolester hydrolase EC 3.1.1.3), cholesterol esterase (sterol ester hydrolase EC 3.1.1.13), phospholipase (EC 3.1.1.4) and of carboxylic ester hydrolases (EC 3.1.1.1) has been studied using isopycnic gradient centrifugation of rat pancreatic homogenates. The identity of the fractions obtained has been established using phase contrast and electron microscopy. Zymogen granules equilibrated at a mean density of 1.224 in aqueous sucrose gradients, mitochondria and cytomembranes at a mean density of 1.19 and 1.16—1.14 respectively. The zymogen fraction accounted for 25—45 % of the total activities of lipase, cholesterol esterase and phospholipase. The specific activities (activity per mg protein) in the zymogen fraction were 5—7 times higher than those of the other cell fractions. A bile salt stimulated carboxylic ester hydrolase was found concentrated to the zymogen granules. The membrane and cell sap fractions accounted for almost all of the remaining lipolytic enzyme activities.

It is well established that pancreatic zymogen granules enclose precursors of proteolytic enzymes as well as amylase and enzymes hydrolyzing nucleotides (Palade *et al.* 1962, van Lancker and Holtzer 1959, Green *et al.* 1963, Keller and Cohen 1961). Furthermore evidence has been presented by several investigators that lipase (glycerol ester hydrolase EC 3.1.1.3), phospholipase (EC 3.1.1.4) and cholesterol esterase (*sterol ester hydrolase EC 3.1.1.13*) are present in the exocrine pancreas and pancreatic juice in different animal species (Desnuelle 1961, Marchis-Mouren 1965, van Deenen and de Haas 1966, Hernandez and Chaikoff 195 , Murthy and Ganguly 1962). Also enzymes hydrolysing water soluble carboxylic esters, designated as carboxylic ester hydrolases (EC 3.1.1.1) have been found in the pancreatic tissue (Sarda and Desnuelle 1958, Mattson and Volpenhein 1966). Reported results from studies of the relationship of lipase to the subcellular components of the pancreas are inconsistent. Hokin (1955) found lipase concentrated in purified zymogen fractions

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from dog pancreatic homogenates. On the contrary Hansson (1959) reported low lipase acti ities in zymógen granules fractionated from the guinea pig pancreas. Recently Seligman *et al.* (1966) using histochemical techniques with both light and electron microscopy found no lipase staining inside zymogen granules in the rat pancreas. *No reports concerning the intracellular localization of phospholipases and chol*esterol esterase in the pancreas have been found.

The investigations reported here were undertaken in order to study the localisation in the pancreatic cell of the lipolytic enzymes, which ha e been found in the pancreatic juice.

Rat pancreatic tissue was fractionated into its subcellular components using isopycnic gradient centrifugation (de Duve 1964)

The identity of the obtained fractions has been established by means of phase contrast and electron microscopy

Materials and Methods

Chemicals. All chemicals used were of reagent grade purity All solvents were redistilled. The chloroform was stabilized with 1.5 % methanol.

Triolein (BDH) was purified on alumina columns (activity grade III according to Brockmann) from which it was eluted with 2 % diethyl ether in petroleum ether A chloroform stock solution was prepared and then stored at 4 C in the dark.

Cholesterol oleat (BDH) was purified on alumina columns (activity grade IV) using 15 % benzene in petroleum ether as the eluent. A heptane stock solution was prepared, and stored t 4 C in the dark.

O elecithin was purified from egg phosphatides (AB V trum, Stockholm) on activ ted silicic acid (Malinckrodt, 100 mesh) columns using chloroform methanol 7 3 as the lvent. A chloroform stock solution was prepared, and stored at −20 C the dark.

Repeated thin layer chromatography of the purified lipids on silicic acid using hexane-diethyl ether acetic acid 83 16 1 and according to Skipski *t al.* (1964) indicated no contamination ben stained with iodine and phosphomolybdic acid.

β-naphtyl acetat was recrystallized from an ethanol solution to giv product free of naphthol.

Sodium taurocholat was synthesized according to Norman (1955) as modified by Hofmann (1964) The purity was controlled by methods described by Hofmann (1964)

Bovin alb mi was purchased from AG Serva, Heidelberg, Germany

None of the substances used contained free fatty acids, as measured by titration according to Dole (1956)

Buff rs 0.1 M glycyl-glycine-NaOH and Tris-HCl buffers were used. Final pH adjustments ere made with pH meter using combined electrode (Type GK 2021C, Radiometer Copenhagen)

Methods

Protein was measured according to Lowry *et al.* (1951) as modified by Eggstein and Kreutz (1955) and lecithin phosphorus according to Chen *et al.* (1954) Triolein glycerol was determined according an Handel and Zilversmit (1957) Cholesterol oleate was measured by the method of Webster (1962) Free fatty acids were titrated according Dole (1956) as modified by Trout *et al.* (1960

Enzyme assay

Lipase, cholesterol esterase and phospholipase activities were estimated by following the release of free fat y acids occurring during hydrolysis of triolein, cholesterol oleate and ovolecithin, respectiv ly The amoun of enzyme the reaction times, pH, temperature, substrat and bile salt concentra ions were chosen t giv linear hydrolysis rates (Fig 1) Carboxylic ester hydrolase activities were determined according to Nachlas and Blackburn (1958 with substitution of 0.05 M Tris-HCl buffer pH 7 4 for eronal buffer

L pase assay system

The ncubation mixture ontained m lume of 1 ml 0.1 ml fraction to be tested, 8 µmoles triolein, 8 µmoles sodium taurocholate nd 5 mg bovine albumin in 0.0? M glycyl-glycine buffer pH 5.

Chol sterol st se assay syst m

I an ttempt to obtai suitable substrat for holesterol esterase assay w has tried dif ferent method for its preparation Murthy nd Ganguly 1962, Mattson and Volpenhein 1 66) *Following the suggestion of Borgstrom (1967) and Mattson and V lpenhein (1966)* w has included octadecane in the substr t mixture Octadecane was found not to inhibit the enzyme and also t be uitable ent in which holesterol oleate easily dissol es. It lso facilitates emul ification

The incubation mixture contained in a olume of 1 ml 0.1 ml fraction to be tested, 16 µmoles holesterol oleate 4 mg octadecane 3? µmoles sodium taurocholate and 5 mg bovine albumin 0.05 M gl lyglycine NaOH buff pH 9.0

Ph pholipase assay syst m

The inc bation mixture ontained in olume of 1 ml 0.1 ml f action to be tested 2 µmoles ovolecithi 4 µmoles sodium taurocholate and 5 mg bovine albumin in 0.05 M glycyl-glycine NaOH buffer pH 9.0

Befor addition of albumin all subs rate solutions ere sonic ted during cooling in Bran son, Stamford Con USA model LS 75 sonifier w th exponential microtip and the power lever ontrol swit h on 7 Sonication times for triolei holesterol leate and ovolecithin sub tr te sol tions wer 1 3 nd 1 min respectiv l A maximal temperat re of 40 C as reached t sonication. Album was added as soon as the mixtures had ooled dow oom empe ature

I bation

An aliquot of the enzyme sourc was taken (maximall 1 mg t sue wet weight f the lipase and holesterol es erase says and 10 mg the phospholipase assay After temperature equi libration for 10 mi t 25 C the appropriate amount of subst t solution was added nd in ion ontinued f r exactly ms t 25 C he lipase nd holesterol esterase assay for mi t 5 C he arbox lic ester h drolase assay and for 2 hours t 38 C in he phos pase assay The enzyme reactions for lipase cholesterol ester se and phospholipase ere ped b adding 4 ml isopropanol 3 N sulphuric acid 40 1 and the leased f ee fatty acids re ed Incubated and nonincubated enzyme nd subst te ontrol er ru in parallel to all says The amou t of fa acids le sed from he enzyme sou was negligable in all lipase and le les er l es rase says while in he phosphol pase assay it had be subt acted. A maximal lease of f ty acids from the enzyme sourc of 30 % of the total releas as re corded Th alue did not interfere w h the res linear lationship bet een the amount of enzyme sou ed activity F g 1

The estal lished pH optima for holesterol ester se and phospholipase ar ac ordanc ith earlier findings Mayer et al 196 M rthy and Ganguly 196 The pH optimum of 7.5 for pancrea ic lipase n l l-gly ne buffer and with hi h oncent tions of bile salt n different from that eported l Borgstrom 1954 who found pH-optimum of 6 7 hen using phosphate buffe and pu ic pan lipase This an be due t he eported Green et al. 1 63 Hokin 19 Siekevitz nd Palade 1 58 omplete breakdow of zymogen granules onl t pH low H uld indic te tha lipase low pH remains partially enclosed inside the zymogen gr nule and is not di ectly ailable f r it substr t the pancre tic lipase Lipase activit ther an reliable the absen of bile sal nd increased th increase of bile salt oncentr n he nc ba ion mi ferent pancre ic holesterol ester se and phospholipase wer inac he absence of bile salt Fig 1

No effect of sucrose or n or esponding to hose used in he gradien was observed Measurement of the enzyme ies differen ime int rv l after the homogeni tion 1 6 hrs gav no differ es

P paration f su se gr die

Linear sucrose concentration adie exten ing from density of 1.12 t 1 1 ere prepared in 5 ml ellulose nit tes Beckma ms syringe stem, ccording t Brak and Lang (19) As evidenced b on rol experiments, the gradient did not hange during centrifugation (F 1) made it possible in every experiment to e non central and reference gradient pr pared xactly e same w y the tes gradients xcept for layering on top of the gradients 1 ml M sucrose solution instead of pancrea pr

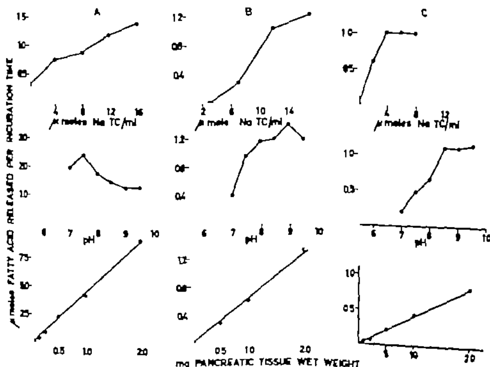


Fig. 1. The influence of bile salt; pH and enzyme concentration on lipase (A) cholesterolester [illegible] (B) and phospholipase- (C) activities in [illegible] pancreatic homogenates. (For det[illegible] see Metho[illegible] [illegible] Results.)

[illegible]stant. The reference gradients were emptied [illegible] the same drop fractions (see preparation of homogenates and fractionation) as the test gradients, and the fractions were used f[illegible] [illegible] of refractiv[illegible] indices [illegible] an Abbe [illegible]efractometer. The sucrose concentration of each f[illegible]action wa[illegible] determined by reference to [illegible] standard cur[illegible] relating concentration to ref[illegible]active index (T[illegible]ble I) and the corresponding densities were derived from data presented by de Duve [illegible] (1[illegible])

Pr[illegible]paration [illegible] homogenat[illegible] [illegible]d [illegible] [illegible]ctionation

Male Sprague-Dawley rats (AB Anticimex, Stockholm, Sweden) wei[illegible]hing 220 [illegible] 280 g were fasted, but had free access to tap wa[illegible]er from 8 am until 4 am the next day. The [illegible] then were killed by a blow on their heads and immediately thereafter brought into the [illegible]old room (+4 [illegible]C) where all subsequent steps in preparation and fractionation of homogenates were performed. The pancreas was excised as quickl[illegible] as possible. After pooling [illegible] [illegible] pan[illegible]creatic tissue from 3—5 animals 0.25 M sucrose [illegible] added to [illegible] dilution [illegible] 1 gram [illegible] weight in [illegible] total [illegible] of 5 ml. Homogenization then consisted of 5 down-u[illegible] strokes [illegible] teflon pestle (abou[illegible] 0.05 mm clearence in the tube all around) inserted in [illegible] homogenizer (Tri-R Instruments, Jamaica, N.Y. USA) with the motor adj[illegible]sted to [illegible] (approx. 2000 rpm). The homogenate was filtered through one layer of gauze and then [illegible] fuged in [illegible] cold Servall centrifuge [illegible]t 500 × g_{av} for 10 min. The [illegible] [illegible] from [illegible]he sediment, which contained cell debris, nuclei and [illegible] [illegible]mall amoun[illegible] [illegible] granules as observed in the phase contrast microscope.

0.4 ml of the resulting supernatant was caref[illegible]lly layered on top of the [illegible] gradients and then cen[illegible]rifuged [illegible] 135,000 × g_{av} for 2 hrs in [illegible] [illegible] TC, using [illegible] 3 × 5 ml [illegible] [illegible]out head. (The temperature measured [illegible] [illegible] did not exceed 7 [illegible]C). The tubes were emptied by piercing the bottom usin[illegible] [illegible]nd 15 drop fractions (approx. 0.375 ml) were collected in preweighed [illegible]

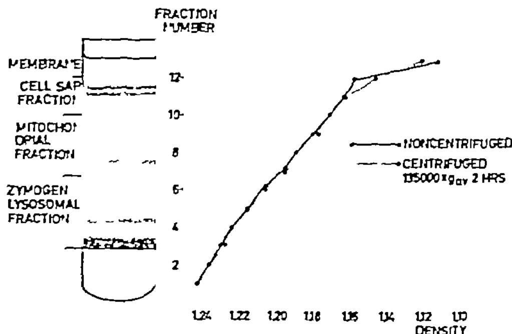


Fig. 2. Distribution of subcellular particles from rat pancreatic homogenates in gradient tube after preparative centrifugation, compared [illegible] individual fraction densities in centrifuged and noncentrifuged reference gradients.

TABLE 1 Properties of [illegible] sucrose reference gradients. [illegible] centrifuge tubes containing sucrose gradients were [illegible] the bottom and [illegible] drop fractions collected, weighed and refractive [illegible] on [illegible] Abbe refractometer (for details see methods). The [illegible] express the means of [illegible] gradient determinations ± SD

Fraction number	Weight in gm		Refractive index		Density (20° C)	
1	[illegible]4	0.0[illegible]	1.4703	0.0007	1.24 ±	0.002
	[illegible]4	0.03	1.418[illegible]	0.00[illegible]	1.[illegible]	0.00[illegible]
3	0.4[illegible]	0.01	1.41[illegible]	0.0008	1.230 ±	0.00[illegible]
4	[illegible]4	0.01	1.4140	0.0009	1.224	0.00[illegible]
	[illegible]4	0.02	1.411	0.0009	1.216	0.00[illegible]
	[illegible]4[illegible]	[illegible]03	1.40[illegible]3	0.0009	1.208	0.003
	[illegible]4[illegible]	[illegible]01	1.40[illegible]1	0.0010	1.19[illegible] ±	0.003
8	0.4[illegible]	[illegible]1	1.4[illegible]19	0.0010	1.190	0.00[illegible]
9	[illegible]43	[illegible]1	1.3948	0.[illegible]010	1.181	0.003
10	0.4	[illegible]	1.3[illegible]1	0.0011	1.1[illegible]2	0.003
11	0.4	[illegible]1	1.3[illegible]	0.00[illegible]	1.1[illegible]	0.0[illegible]4
1[illegible]	0.4	[illegible]1	1.3[illegible]9	0.[illegible]011	1.1[illegible]	0.004
13	0.4	[illegible]12	1.3[illegible]4	0.01[illegible]	1.114	0.018

Preparation for fraction identification

Fractions with the same density from 3 separate testgradients were pooled and then fixed for 15 min in 1 : 1 (v/v) 2 % glutaraldehyde followed by 1 : 1 (v/v) 2 % osmium tetroxide for 3 hrs. The glutaraldehyde and osmium tetroxide were dissolved in sucrose solutions of a density similar to the density of the fraction to be fixed. After transferring to 3 ml cellulose nitrate tubes with a plane hard agar-agar layer (2 % in 2.0 M sucrose) at the bottom the fixed fractions were centrifuged for 20 min at $20,000 \times g_{av}$ in a swing out head. The supernatants were decanted and 2 % liquid agar-agar was layered on top of the plane pellets. After hardening of the agar-agar the pellets were cut out and dehydrated in increasing concentrations of acetone. Representative parts of the pellets were then embedded in Vestopal W and after sectioning identified by means of electron microscopy.

The preparation of the pancreatic fractions for electron microscopy was carried out in this laboratory following methods suggested by Sjöstrand (1967). The sectionings and the preparations of the electron micrographs of the different fractions were performed by Sjöstrand.

The proportion of the total particle volume occupied by the different particle populations in each pancreatic subfraction were determined by means of the method described by Sitte (1965).

Results

A distribution of the tissue components, depicted in Fig. 2 was observed after centrifugation of the test gradients.

Fraction identification

The isolated fractions were examined in the light microscope, under phase contrast optics and in the electron microscope.

Fig. 3 shows electronmicrographs of the pancreatic subfractions. Obviously the particle populations were not completely separated, but the fractions with the mean equilibrium densities of 1.22, 1.19 and 1.17—1.148 contained mainly zymogen granules, mitochondria and cytomembrane structures respectively. Free ribosomes were present almost entirely in the fractions with low densities near but below the interphase between the gradient and the superlayered 0.25 M sucrose. The uppermost fraction contained mainly an amorphous material but also small quantities of free ribosomes and rough surfaced membranes. Smooth surfaced membranes were sparsely represented in those fractions where zymogen granules and rough surfaced membranes were abundant. Very small numbers of lysosomes appeared in the zymogen granule fractions.

Fig. 4 shows the relative volumes occupied by the different particle populations in each of the test gradient subfractions.

In some experiments we have observed an aggregation of unidentified material at high density levels. The equilibrium levels for the individual particle populations did not alter when centrifuged in the gradient using different centrifugal forces (5000 and $10,000 \times g$) and times (2 and 4 hrs). However in this investigation the high speed was chosen for better resolution at the equilibrium levels. The results therefore also indicate that isopycnic equilibrium for the cell components have been reached.

Biochemistry

Because of the morphological heterogenity in the sediment we have excluded its protein and enzyme content from our calculations. Thus, the recoveries have

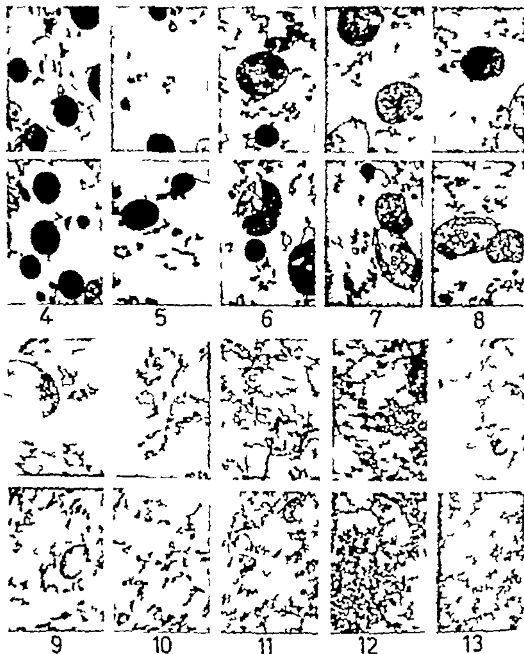


Fig. 3. Electronmicrographs of [illegible] *bcellular* [illegible] *actions obtained after isopycnic centrifug*[illegible]*ion of ra*[illegible] pancreatic homogenates. The micrographs [illegible] ere prepared from sections at different levels of the pellets, obtained by centrifugation [illegible] of the fixed isolated pancreatic subf[illegible]tions. Total magnification 4 000 of f[illegible] 4 [illegible] 8 and 21 000 of fractions 9–13. *For further* details see Methods.

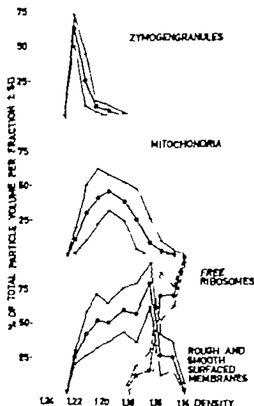


Fig. 4 Distribution of the proportion of the total particle olume occupied by the subcellular particle populations after isopycnic centrifugation of rat pancreatic homogenates. (The relative olumes were estimated by means of the method described by Glte (1966) The plotted data represent the mean ± SD of at least 12 9 × 9 cm lewfields on electronmicrographs from sections t different levels of the pellets obtained by centrifugation of the fixed pancreatic subfractions.)

calculated only with reference to the supernatant which has been applied to the test gradients. A maximal loss of 10 % of the enzymic activities to the sediment was observed.

Protein distrib tion

A mean protein content per gram of tissue wet weight of 164 ± 30 mg was found. The supernatant obtained after the first centrifugation at 500 × *g* for 10 min had a mean protein content of 140 ± 23 mg/g of tissue wet weight. After centrifugation of the supernatant applied to test gradient the protein was distributed according to Table II and Fig 5 The good recoveries of protein from the test gradients (about 90 %) indicate that the adopted way of determination of densities of the fractions is adequate, and also that the cell constituents separated during th centrif gation procedure have reached isopycnic equilibrium.

Int acellular dist b tio f lipolytic nzym

Fig 5 shows the distribution of lipase activ ty n the test gradients. Lipase ctivity forms two distinct peaks this is at a mean density of 1 224 and 1 156, corresponding to the zymogen granule nd to the membrane-cell sap fractions respectively About 40 % of the total activity is found in the zymogen granule fractions and 45 In th

TABLE II Per cent distribution and recovery of protein nd lipase holesterol esterase nd phospholipase activities after eq ilibrium density centrifugation of rat pancreatic homogenates.

Measured	Applied	Recovered	Recovery %
Protein	10.8 ± 2 1	10.2 ± 1 7	93.3 ± 4 1
Lipase	234.2 ± 32.2	189 0 ± 45 1	80.1 ± 2.7
Cholesterol esterase	23.9 ± 4 4	19 0 ± 2.3	80.0 ± 7.6
Phospholipase	4 8 ± 1.3		

membrane-cell sap fractions (Table II) However due to some losses of zymogen granules to the sediment, the figures obtained for the zymogen fractions probably should be even somewhat higher The small and diffusely distributed activity in the mitochondrial fractions may be ascribed to a contamination by zymogen granules or membranes.

The specific activities indicate that in the zymogen fractions the enzyme is about 5—7 times concentrated compared to the membrane fractions and the applied supernatant.

Cholesterol esterase revealed a pattern of distribution within the pancreatic subcellular fractic ns similar to that of lipase (Fig 5 and Table II) However th membranes nd cell sap contained a somewl at higher (about 10 % rel.) fraction of the total enzyme actn ty The zymogen fraction shows the highest specific activity (Fig 5) indicating a 5-fold concentration of enzyme activity in these particles as compared to the membrane cell sap fraction, and a 2.5-fold concentration compared to the supernatant layered on top of the test gradients.

The preparation had very low hydrolytic activities against ovolecithin. Nevertheles this activity was localised in th zymogen and membrane-cell sap fractions However in the membrane ell sap fractions there was extensive release of fatty cid in the absence of added ubstrate resulting in high blank alues (>30 %) The measured activity was theref re unreliable In the zymogen and mitochondrial fractions the hydrolysis in the absenc f added substrate was negligable compared to hydrolysis in it presence We therefore present experimental data only for the zymogen and mitochondrial fractions (Tabl II) About 25 of the phospholipase activity n the supernatant was found in the zymogen fraction. A better method for studying phospholipase activity in the membrane ell sap fractions is needed and the use of labeled substrates might prove adequate Prel minary experiments with β- H-acyl labeled lecithin as a substrate show that mainly the β fatty acid is hydrolyzed by nzymes present in th zymogen fraction. In t mitochondrial fractions no hydrolysis ld be demonstrated either in the presenc r absence of added substrate This re lt in

Homogenates from overnight fasted rats containing 1 g tissue wet eight in 5 ml 0.25 M sucrose were centrifuged at 500 × g_{av} for 10 min. 0.4 ml of the resulting supernatant was pplied t tube containing the aqueous sucrose gradient and then centrifuged in a swing out head for 2 hrs t 135 000 × g_{av}

Per cent recovered t densities			E pressed as
1.242—1.208	1.199—1.181	<1.170	
13.8 ± 3.1	12.0 ± 2.5	72.5 ± 6.0	mg
41.2 ± 1.4	12.5 ± 3.5	46.1 ± 6.8	
32.9 ± 4.1	11.5 ± 0.5	55.6 ± 4.1	μmoles fatty acid released per incubation time
26.2 ± 1.8			Per cent of applied activ ty

the pancreas therefore does not confirm Björnstad's studies (1966) with liver mitochondria in which it was shown that extensive hydrolysis of endogenous substrate occurred.

Carboxylic ester hydrolase activity in the different fractions was tested using β naphthyl acetate at concentrations which rendered a completely water soluble substrate. Activities were measured in the presence and absence of bile salt (16 μmoles sodium taurocholate in 5 ml ncubation mixture) The distribution of these activities is shown in Fig. 6. In the absence of bile salt all fractions were capable of spl tting β naphthyl acetate to an extent which essentially followed the protein content of the fractions, and no peak of activity in the zymogen region was found

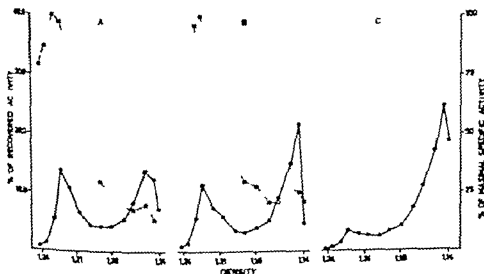


Fig. 5 Distribution of total and specific activities for lipase (A) cholesterol esterase (B) and of protein (C) after isopycnic gradient centrifugation of rat pancreatic homogenates.
●——● Total activity or amount. □- - -□ Specific activity (The data represent the means in at least four experiments)

When tested in the presence of bile salt, however, hydrolysis of β naphthyl acetate appeared in two definite peaks, one confined to the zymogen granule fractions (3—4-fold increase) and the second to the membrane cell sap fractions (2—3-fold increase).

Discussion

The experiments reported here indicate that a relatively pure zymogen fraction, consisting of intact zymogen granules can be isolated from homogenates of the rat pancreas by isopycnic centrifugation in linear aqueous sucrose gradients. This method, however, can only serve analytical purposes, but it offers a possibility of separation of other subcellular components in a relatively pure form in a single centrifugation procedure.

The zymogen granules have been found to equilibrate in the gradients at a density somewhat higher than liver lysosomes as reported by de Duve *et al.* (1961), but not at as high density level as suggested by Siekevitz and Palade (1959). No attempt was made to separate the membrane fractions into more detailed structures and thus the precise localization of the enzymes within these fractions cannot be assessed. It has, however, been suggested (Palade *et al.* 1962) that enzymes secreted into the pancreatic ducts are produced by ribosomes attached to rough surfaced membranes (α-cytomembranes). In addition, it has been suggested by Sjöstrand (1962) that enzymes after being produced by ribosomes or other cell structures are then incorporated into precursor granules which derive their membranes from the Golgi apparatus. Our membrane fractions presumably contain these structures.

In view of the results obtained in this investigation, it can be concluded that high proportions of the lipase, cholesterol esterase and phospholipase activities are localized to the zymogen granules of the rat pancreas. This conclusion is based on the fact, that in overnight fasted rat around 25—45 % of the total enzyme activities are

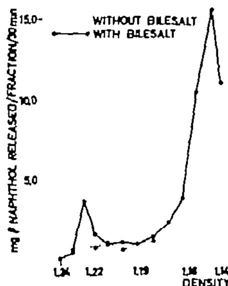


Fig. 6. Total carboxylic ester hydrolase activity distribution after isopycnic gradient centrifugation of rat pancreatic homogenates.

present there, and that the specific activities show a 5—7 fold concentration of the said enzymes in the zymogen granule fraction as compared to the other cell components. It also is evident, that all the enzymes are present in the membrane fractions.

The intracellular distribution of lipase in the rat pancreas demonstrated in the present study is in agreement with the findings of Hokin (1955) with respect to its localisation in zymogen granules. The fact, that Hansson (1959) who used guinea pig pancreas could not demonstrate lipase as being concentrated in the zymogen fraction could probably be due to species differences. The disagreement between our findings and the histochemical evidence for lipase localisation in the rat pancreas obtained by Seligman *et al.* (1966) who incubated pancreatic slices with an aromatic thioester of nonanoic acid, may only be apparent. In that case it would mean, that lipase is attached to the outer surface of the zymogen membrane. But it also could be due to the fact, that in the conditions used in their study the substrate was not accessible for the enzyme residing inside the granules and the histochemical reaction did only occur at the site of contact between the substrate and enzyme, that is outside, but close to the zymogen membrane.

The bile salt stimulated carboxylic ester hydrolase shows a similar distribution to that of the other lipolytic enzymes, with two peaks of activity—one in the zymogen granule—and one in the membrane cell sap region. According to the evidence presented by Morgan *et al.* (1967) for rat pancreatic juice, this activity may be representative for a single nonspecific enzyme hydrolyzing also cholesterol esters and monoglycerides when present in micellar solutions. However in the absence of bile salt the pattern of distribution of activity against β naphthyl acetate did not show a peak in the zymogen fraction. This could indicate a presence in the pancreas of other carboxylic ester hydrolases which are not concentrated in the zymogen granules. The presence of an esterase in the pancreas which is not stimulated by bile salt has already been postulated by several authors (Sarda and Desnuelle 1958, Mattson and Volpenhein 1966, Seligman *et al.* 1966).

The results in this investigation thus provide evidence that all lipolytic enzymes which are secreted into the pancreatic juice have the same intracellular distribution in the pancreas as trypsinogen and chymotrypsinogen (Siekevitz and Palade 1958).

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References

BJÖRNSTAD, P. Phospholipase activity in rat liver mitochondria studied by the use of endogenous substrates. *J. Lipid R.* 1966 *7* 612—620.

BOCK, R. M. and N. S. LING. Devices for gradient elution in chromatography. *Anal. Chem.* 1954 *26* 1543—1546.

BORGSTRÖM, B. Effect of taurocholic acid on the pH-activity curve of rat pancreatic lipase. *Biochim. biophys. Acta* (Amst.) 1954 *13* 149—151.

BORGSTRÖM, B. Personal communication.

CHEN, P. S. Jr., T. Y. TORIBARA and H. WARNER. Microdetermination of phosphorus. *Anal. Chem.* 1956 *28* 1756—1758.

v. s. Dee. ex, L. L. M. and G. H. de Haas. Phosphoglyceride and phospholipases. *A. n. Rev. Biochem.* 1966, 35, I, 157—194.
Desnuelle, P., Pancreatic lipase. *Ad. en. E. zymol.* 1961, 23, 129—161.
Dole, V. P. A relation between non-esterified fatty acid in plasma and the metabolism of glucose. *J. lin. I. st.* 1956, 35, 150—154.
deDuve, C., The separation and characterization of subcellular particles. *Harvey Lect.* 1964, 59, 44—87.
deDuve, C. J. Berthet and H. Be. u. e. v. Gradient centrifugation of cell particles: theory and applications. *Pr. gr. Bi. phy.* 1959, 9, 326—36.
Dootter, M. and F. H. Kreut. Vergleichende Untersuchungen zu quantitativen Eiweissbestimmung in Liquor und eiweissarmen Lösungen. *Kli. W. sch.* 195?, 33, 879—881.
Greene, L. J. C. H. W. Hirs and G. E. Palade. On the protein composition of bovine pancreatic zymogen granules. *J. biol. Chem.* 1963, 238, 6, 2054—20 0.
v. H. ndel, E. nd D. B. Zilversmit. Chemical determination of triglycerides. *J. lin. M. d.* 1957, 50, 152—158.
H. son, E. The formation of pancreatic juice proteins studied with labelled amino acids. *Act. physiol. sc. d.* 1959, 46, Suppl. 161.
Hernandez, H. H. and J. L. Chaikoff. Purification and properties of pancreatic cholesterol esterase. *J. biol. Chem.* 1957, 228, 447—457.
Ho. M. A. F. *Th. f. tion. f bile salt. fat. bsorption.* Thesis, Lond. 1961, pp 5..
Hokin, L. E. Isolation of the zymogen granules of dog pancreas and a study of their properties. *Biochim. biophys. Act.* (Amst.) 1955, 18, 379—388.
Keller, P. J. and E. Cohen. Enzymic composition of some ... ll fractions of bovine pancreas. *J. Biol. Chem.* 1961, 236, 5, 1407—1413.
L. ker, J. L. and R. L. Hoeltje. Tissue fractionation studies of mouse pancreas. *J. biol. Chem.* 1953, 234, 2359—2363.
Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. biol. Chem.* 1951, 193, 265—27.
Magee, W. L., J. Gallai-Hatchard, H. S. ... and R. H. S. Thompson. The purification and properties of phospholipase A from human pancreas. *Biochem. J.* 19.. 83, 1—5.
Marchis-Mouren, G. Étude comparée de l'équipement enzymatique du suc pancréatique de diverses espèces. *Bull. Soc. Chim. biol.* Paris 1965, 47, 2207—221.
M. rtson, F. H. and R. A. Vahouny. Carboxylic ester hydrolases of rat pancreatic juice. *J. Lipid R.* 1966, 7, 536—545.
... pro R. G. H. J. Barrowm ... H. Filip. Wender and B. Borgström. The lipolytic enzymes of ... pancreatic juic. *Bioch. m. bi. phy. A. t.* Amst. 1967, 146, 314—316.
Ii. tty S. K. and J. G. ... Studies on cholesterol esterases of the small intestine and pancreas of rats. *Biochem. J.* 196.. 83, 460—469.
... M. M. and R. Black. The colorimetric determination of urinary lipase. *J. biol. Chem.* 1958, 30, 1051—1061.
Norm. A. Preparation of conjugated bile acids using mixed carboxylic acid anhydrides. *A. t. ... K. m.* 1955, 8, 32, 331—312.
Palade, G. E., P. Siekevitz and L. G. Caro. Structure, chemistry and function of the pancreatic exocrine cell. I. *C. ba Fou. d. on Symposium on the ... exocrine pan. ... J.* and A. Churchill, Ltd. London 196.. 23—55.
Sarda, L. and P. De. ... Action de la lipase pancréatique sur les esters en émulsion. *Biochim. biophys. A. t.* Amst. 1 58, 30, 513—521.
Seligman, M. L., H. U. ... J. S. H. ... ker, S. P. ... H. W. ... and A. M. Seligman. Cytochemical localization of pancreatic lipase with light and electron microscope. *E. pl. and Mol. Pathol.* 1966, Suppl. 3, 1—33.
Siekevitz, P. and G. E. Palade. A cytochemical study on the pancreas of the guinea pig. *J. biophys. biochem. Cytol.* 1 58, 4, 2, 203—17.
Sitte, H. Morphometrische Untersuchungen an Zellen. *Symp. quant. Method. Morphol.* 8 Anat. Kongr. Wiesbaden 1965. Springer Verlag 196.
Sjöstrand, F. The fine structure of the exocrine pancreas cells. I. *Ciba Foundation Symposium on th... exocrine pa...* J. and A. Churchill, Ltd. London, 1962. 1—19.
Sjöstrand, F. Personal communication.
Skipski, V. P., R. F. Peterson and M. Barclay. Quantitative analysis of phospholipids by thin-layer chromatography. *Bioch. m. J.* 1964, 90, 374—378.
Trout, D. L., E. H. Estes and S. Friedberg. Titration of free fatty acids of plasma: study of current methods and new modification. *J. Lipid Res.* 1960, 1, 199—202.
Webster, D. The determination of total and ester cholesterol in whole blood, serum or plasma. *Clin. him. Act.* 1962, 7, 277—284.

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Changes in Dynamic Sensitivity of Primary Endings of Muscle Spindle Afferents Induced by DOPA

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Abstract

Bergmans, J and S. Grillner. *Change in dynamic sensitivity of primary end ng of muscle pindle afferent indu ed by DOPA* Acta physiol scand 1968. *74* 629—636.

In the spinal unanaesthetized cat, muscle spindle afferents from primary endings originating in the tenuissimus muscle wer studied during ramp stretch to the muscle with intact efferent nerv supply before and after an intravenous injection of DOPA. It as demonstrated that the peak and mean frequency during stretch was decreased after DOPA, when there also occurred firing during elease of stretch. The results indicate that there is after DOPA decreased spontaneous activity in dynamic γ-motoneurones and concomitant increase in static fusimotor activ ty The findings support the idea that descending noradrenergic fibres control in reciprocal fashion, the spontaneous activ ty in static and dynamic γ-motoneurones to flexors.

Matthews (1962) demonstrated the existence of two types of γ motoneurones, static and dynamic, which act in an opposit way on the dynamic sensiti t of muscl spindle primary endings. Hunt (1951) and Hunt and P ntal (1958) ha e shown that part of the motoneu ones are pontaneously activ n the pinal cat This fraction is probably identical w th dynamic motoneurones sinc Alnaes, Jansen and Rudjord (1965) ha shown a decrease of dynamic sensit t of muscl spindle primary endings when d -efferent ng flexo muscl spindles n spinal preparation, but no sign of a changed st tic a t t on de-efferentation However after an injection of DOPA n th pinal preparation Grillner H ngr nd L ndberg (1967) found an increased act t n pindle fferent from sec ndar endings This was interpreted as caused b n nc eased activ t n tatic motoneu ones since secondary endings nl nfl enced f m static motoneu ones (Appelberg Bessou and Laport 1966 Wh ecording the activ ty ngl -eff ents n the spinal prepa tion Berg ans and Grillner 1967 found tw types f -efferents which wer influenced in q t differen manner by an njectio f DOPA In the first type th resting activit decreased he other ne h es ng activ t n- creased fter DOPA On the basis of the f nd ngs l Alnaes al (1965 nd

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Grillner *et al.* (1967) it was suggested that the first type is identical with dynamic and the second with static γ-motoneurones. This hypothesis has now been further tested by investigating the response of muscle spindle afferents from primary endings to linear stretch of different velocities before and after an i.v. injection of DOPA.

This investigation is particularly concerned with possible changes in dynamic fusimotor activity after DOPA, since it is of utmost importance for the above suggestion to know whether the activity in dynamic fusimotor fibres is influenced by an i.v. injection of DOPA.

Methods

Experiments were performed on 14 cats anaesthetized with ether during dissection and then intercollicularly decerebrated and spinalized at lower thoracic level. The tenuissimus muscle of the left hindlimb was dissected free with intact nerve and blood supply. The biceps muscle was removed. The sciatic nerve with branches (except tenuissimus), the inferior and superior gluteal, the ischio-pubofemoral nerve and other branches in the gluteal region were cut on the ipsilateral side (Bessou and Laporte 1 51). Laminectomy was performed from L3 to L7 and a smaller one at the lower thoracic level for spinalization. To prevent the movement that often occurs after an i.v. injection of DOPA, but at the same time leave as much afferent inflow as possible, the ventral roots of L4 to L7 and S1 to S4 were cut on the ipsilateral side and L3 to S4 on the contralateral side. In some earlier experiments the obturator and femoral nerves including the nerve to iliopsoas were cut bilaterally and the sciatic nerve contralaterally. The exposed lumbar spinal cord and the dissected part of the hindlimb were covered with paraffin; the temperature in the paraffin pools and rectum was controlled and kept constant within 36.5–38° C. Blood pressure was recorded continuously and blood loss substituted by a mixture of low and high molecular dextran. The dorsal roots were left intact but for small filaments of L7 or S1 in which afferents from tenuissimus were isolated. The afferents were identified as originating from muscle spindles by the pause during extrafusal contraction (B. H. C. Matthews 1933) and as originating from primary or secondary endings by their conduction velocity. The division between group I and II was chosen at 72 m/sec (Hunt 1 54).

Muscle preparation. After isolation of one or two spindle afferents from primary endings, a segment of 3–4 cm of the tenuissimus muscle containing the spindles in question was isolated (Bessou and Laporte 19 3). The pelvic portion was rigidly fixed and the distal part connected to an electromagnetic puller which allowed linear stretch up to 10 mm at velocities between 1 90 mm/sec. The activity in the dorsal root filament was recorded together with the length displacement of the muscle caused by the puller on a modified Tektronix and photographed on running film by a Grass camera type C4-K. Either the tension recorded by a sensitive strain gauge or the electromyogram plus the activity was recorded continuously.

In the earlier experiments the afferent recording was displayed directly on the latter; the instantaneous frequency (the reciprocal of the interval to the preceding action potential) was displayed on the oscilloscope. To monitor extrafusal activity we recorded the EMG between one electrode on the distal and one on the proximal part of the muscle segment under investigation. This is a more sensitive method to register extrafusal activity than the tension recording via a sensitive isometric strain gauge, since the muscle fibres in the tenuissimus muscle are arranged in series (Adrian 19 5). Due to the marked elasticity of the muscle the contraction of a single motor unit in one segment gives only a very moderate increase in tension.

The afferent activity was studied during resting conditions and during stretch and release of stretch at different velocities before and after an i.v. injection of L-DOPA (l-3,4-dihydroxyphenylalanine) (50 10 mg/kg) and in some experiments also after de-efferentation of the muscle following transection of the ventral roots L and S1 or after Flaxedil® (gallamine triethiodide) injected (Hunt 1 52). The completion of the intrafusal block after Flaxedil was checked by pinching the abdominal wall just in front of the hip bone, which accelerated the ending markedly in the intact preparation both before and after the injection of DOPA. This manoeuvre was effective also when the extrafusal but not yet the intrafusal block was complete.

Terminology. The term fusimotor activity will be taken to indicate any efferent control of the spindle independent of whether in γ- or slow α-fibres (P. Matthews 1 4). In consequence, dynamic fusimotor activity denotes an efferent activity giving an increased dynamic sensitivity of primary endings.

Results

1) *The dynamic sensitivity before and after an intravenous injection of DOPA*

The dynamic sensitivity of muscle spindle afferents from primary endings can be investigated by applying linear stretch to the muscle under investigation. For the present purpose, i.e. differentiating dynamic and static fusimotor activity it was difficult to employ the dynamic index (Jansen and Matthews 1962, Crowe and Matthews 1964a) which is the difference between the peak frequency at the end of the stretch and the frequency 0.5 sec after completion of the stretch. The reason is that static fusimotor activity will by itself reduce the dynamic index. However static fusimotor activity never decreases the peak or mean frequency (Crowe and Matthews 1964a, Brown and Matthews 1966).

Fig. 1 is from a muscle spindle afferent from a primary ending originating in tenuissimus (*cf.* Methods). The instantaneous frequency is shown on the ordinate and time on the abscissa. The peak frequency at completion of stretch (2.5 mm) at a velocity of 32 mm/sec is decreased from 290 imp/sec before (A) to 85 imp/sec after injection of DOPA (C). At a lower speed of stretch (7.5 mm/sec) the decrease is as evident (B and D). Records B and D are taken at a slightly lower resting tension. The initial burst of activity at the beginning of the dynamic phase of stretching that can be seen in records B and D has been described by Jansen and Matthews (1962) and Crowe and Matthews (1964a). In the present context it will not be considered but it has recently been analyzed by Schäfer (1967) and denoted the acceleration response.

The effect of DOPA on the peak frequencies of firing of a spindle afferent from a primary ending at different velocities of linear stretch of the muscle is shown in Fig. 2 A. For velocities ranging from 7.5—55 mm/sec there is after DOPA a marked decrease in peak frequency during stretch. Each value in the diagram is the average

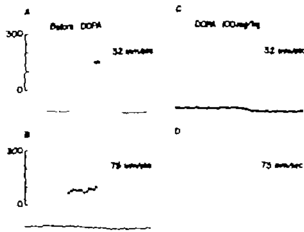


Fig. 1. Changes induced by DOPA in the dynamic sensitivity of a primary ending. The instantaneous frequency (one dot representing one action potential) of a spindle afferent is displayed during a linear stretch (2.5 mm) of the muscle as indicated on the lower beam. The velocity of stretch is indicated at the different records. B and D are taken at a lower resting tension. The left column shows the discharge rate before, the right after an i.v. injection of DOPA (100 mg/kg)

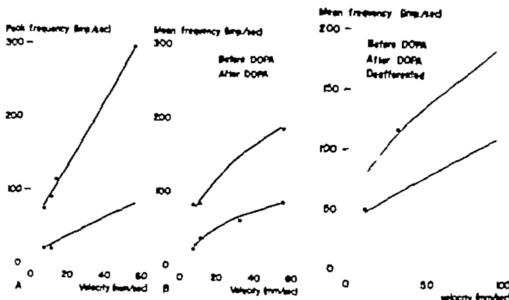


Fig 2 Fig 3

Fig 2 Changes induced by DOPA in peak and mean frequency during stretch. The results are obtained from spindle afferent from primary ending. In A the peak frequency — the frequency of discharge of the afferent just before the end of stretching is plotted versus the different velocities of stretch (abscissa) before (filled circles) and after (open circles) an i v injection of DOPA (100 mg/kg). Each value represents the average of three to five values of the peak frequency obtained during consecutive stretches. In B the mean frequency during the stretching phase is plotted similarly versus velocity of stretch. Each value represents the average of 3–5 values.

Fig. 3 The mean frequency during stretch before and after DOPA and under deefferented conditions. The results are obtained from a spindle afferent from a primary ending. The mean firing frequency during the stretching phase is plotted versus different velocities of stretch before (open circles) and after (filled circles) i.v. injection of DOPA (100 mg/kg) and also after an i v injection of gallamine triethiodide (Flaxedil®) (8.7 mg/kg) which blocks the neuromuscular transmission (cf. Methods) causing a functional deefferentation (crosses).

of the peak frequencies of 3 to 5 different stretches. In Fig. 2 B the corresponding mean frequencies, obtained by counting the number of spikes during the phase of stretching are plotted against velocities of stretch. Also here there is a marked decrease after DOPA, which corresponds to the decrease in 2 A.

Fig. 3 is also from a primary ending. This diagram shows that the mean frequency is decreased after an i. v. injection of DOPA but not further reduced after the functional de-efferentation that was obtained by an i.v. injection of Flaxedil (8.7 mg/kg).

The above results are representative for 6 out of 7 primary endings studied in this way. In the 7th there was a slight decrease in mean and peak frequency at some velocities of stretch but no change at others.

Crowe and Matthews (1964a) have shown that both the mean frequency during stretch and the peak frequency are greatly increased during dynamic fusimotor

stimulation, but only moderately influenced by stimulation of static fibres. The decrease in peak and mean frequency that was observed in 6 out of afferents cannot be due to a decreased static fusimotor activity since this is increased after DOPA (Grillner *et al.* 1967 *cf.* below). Thus it must be due to a decreased activity in dynamic fusimotor fibres. Since the mean frequencies after DOPA (Fig. 3) and in the de-efferent state are very similar the dynamic activity must be decreased to low values after DOPA. It also means that there cannot be a very strong static effect after DOPA in this preparation (see Discussion) since static fusimotor stimulation tends to moderately increase peak and mean frequencies during stretch (Crowe and Matthews 1964a).

It might be significant that the curves that express the relationship of peak (Fig. 2A) or mean (Fig. 2B, 3) frequency versus velocity of stretch is steeper before DOPA (filled circles) than after (open circles). A change of slope can occur with dynamic fusimotor stimulation but does not seem to occur on stimulation of static fibres (Crowe and Matthews 1964a).

In summary, our results indicate that there is, in the spinal unanesthetized animal a resting activity in dynamic fusimotor fibres, which is decreased after an i.v. injection of DOPA.

2) *Release of stretch*

The abrupt silencing of firing on release of stretch was originally described by B. H. C. Matthews (1933) for his A1 type of afferents. Crowe and Matthews (1964b) have shown that on stimulation of static fusimotor fibres, afferents from primary endings fire during release and successively decrease their frequency, while on dynamic fusimotor stimulation the frequency is abruptly decreased to a considerably lower level.

The primary afferent response during release of stretch was used by Alnaes *et al.* (1965) in their studies of naturally occurring γ-bias. In their spinal preparation they had spontaneous dynamic fusimotor activity but no spontaneous static fusimotor activity to flexors since there were no changes in response of group II afferents before and after de-efferentation (Appelberg *et al.* 1966). Release of a standard stretch did not give firing in afferents with primary endings. In the decerebrate preparation, on the other hand, in which static fusimotor fibres exhibit resting activity Jansen and Rudjord (1964) frequently found firing during release of stretch. This firing during release can be looked upon as a sign of static fusimotor activity.

Fig. 4 is a schematic representation of the sweep during release of stretch, each dot indicates the occurrence of one spike in a primary muscle spindle afferent. Each horizontal row indicates one single sweep. Before DOPA the ending fires during release of stretch (9 mm/sec) only in one sweep, but after the injection of DOPA it fires during release of stretch in all the sweeps. This indicates that there is an increased static fusimotor activity after DOPA. The pattern of firing immediately after completion of stretch also shows significant differences.

Under de-efferented conditions the ending of Fig. 4 ceased to fire abruptly

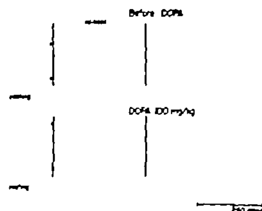


Fig 4 Changes induced by DOPA in the firing pattern during release of stretch of muscle spindle afferent from primary ending This is a schematic representation of five consecutive sweeps each horizontal row indicating one sweep and each vertical bar the occurrence of one action potential. The region between the two vertical bars indicates the release of stretch of 3.2 mm with a velocity of 9 mm/sec The upper series show the firing pattern before and the lower after an i.v. injection of DOPA (100 mg/kg) The resting discharge is illustrated in the lower horizontal row

resumed it activity after completion of release after 3.5 sec (not illustrated) A comparison with the pre DOPA state shows that the afferent resume their activity much sooner thus indicating that the spindle before DOPA is under a fusimotor bias, presumably of the dynamic type

Thus there is evidence for an increased static fusimotor activity in afferents from primary endings after an i.v. injection of DOPA These results are in complete agreement with the result of Grillner *et al* (1967) showing an increased static activity by the increase in resting discharge in group II afferent after an i.v. injection of DOPA

It is difficult to predict what effect a decrease of dynamic bias and a simultaneous increase in static bias should have on the resting discharge of the primary afferents, since it is elevated by stimulation of both static and dynamic fusimotor fibres (Matthews 1962, Crowe and Matthews 1964a, Brown Crowe and Matthews 1965) The conditions under naturally occurring γ-bias may differ from the above since static γ motoneurones are more common than dynamic (Bessou and Laporte 1966 In our experiment we often found a slight elevation of the resting discharge in primary endings after DOPA

Discussion

Activity in dynamic γ motoneurones increases the dynamic sensitivity of spindle afferents from primary endings but this effect is also produced by stimulation of slow *a*-fibres that excite both extra- and intrafusal muscle fibres (Bessou Emonet Denand and Laporte 1965 Thus the decrease in peak or mean frequency that occurs after an i.v. injection of DOPA can be due to a decreased activity in either or both of these two types of fibres Since Barker (1967) has recently claimed that the type of plate endings (P1) that he ascribes to collateral of *a* fibres exists in 58% of the spindles investigated, the possibility of spontaneous activity in these neurones had to be excluded. If there was a tonic slow *a*-fibre activity a continuous extrafusal activity should be encountered in the pre DOPA state This has been investigated

carefully but no sign of continuous electromyographical acti ty was found under resting conditions in tenuissimus. Thus, in agreement with Alnaes *et al* (1965) t can be concluded that under resting conditions in the normal spinal cat there is spontaneous activity in dynamic γ-motoneurones. This activity is decreased to low levels after an i.v injection of DOPA. The firing during release of stretch indicates that there is an increased static activity after DOPA, which confirms the results of Grillner *et al.* (1967)

In the experiments with recording from γ-efferents, Bergmans and Grillner (1967) found a marked acceleration in the efferents tentatively identified as be longing to static γ motoneurones. From their results t may seem surprising that the present investigation only indicated a moderate increase in static fusimotor activity This may be due to the more extensive de afferentation used in the present series (*cf* Methods) Hunt (1951) has shown that the spontaneous γ-activity in the spinal cat is dependent on the afferent inflow Even if Voorhoeve (1958) and Lindsley and Eldred (1961) have shown that a residual spontaneous activity exists in the spinal and de-afferented preparation, it seems likely that the spontaneous acti ity that occurs in the spinal preparation is largely dependent on th dorsal root inflow (*cf* Hunt 1951) These results refer only to dynamic γ-motoneurones, since static are not active in the spinal preparation (Alnaes *et al.* 1965) However it is probable that the afferent inflow is also of importance for the spontaneous activity that occurs in static γ-motoneurones after DOPA, since the γ-efferents tentatively identified as static (Bergmans and Grillner 1967 *cf* below) decreased thei resting activity when the dorsal roots were cut bilaterally (Bergmans and Grillner unpublished)

Hence because of some difference n the experimental procedure the present re sults cannot from a quantitative point of view be compared with those of Bergmans and Grillner (1967) Thei importance is, however qualitative *In th pinal at an jection of DOPA decrease the activity t dynamic γ m t neu nes and increases the activity in static γ-m toneurones*

From these results together with the results of Alnaes *et al.* (1965) and Grillner *et al* (1967) it is evident that the two types of γ-motoneurones that were dif ferentiated on behalf of spontaneous activity and reflex activation before and after DOPA (Bergmans and Grillner 1967) can readily be identified as dynamic, .e spontaneously active before DOPA (type A of Bergmans and Grillner 1967) nd as static, spontaneously ctive only after DOPA (type B of Bergmans and Grillner 1967)

Since the effect of DOPA ery likely is to cause liberation of noradrenaline from descending noradrenergic fibres in the spinal cord (Andén t al. 1966a, Andén, Jukes and Lundberg 1966b) the effect of DOPA should be looked upon as resulting from n activation of the descending noradrenergic system. Thus f th abov nter pretation is correct, this system controls the spontaneous acti ity by depressing the resting activity in dynamic γ motoneurones and at the same time enhancing t l static γ motoneurones. Th functional implications of the present results will be considered n a forthcoming paper

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References

ADRI E. D The pread of activity in the tenuissimus muscle of the at and in other complex muscles. *J Physiol.* (Lond.) 192. 60 301—315.
AL ARA, E J K. S J z and T R DJORN, Fusimotor acti ty in the pinal at. *Act phy siol. sc nd.* 1965 63 19 — 12
ANDE N E, M. C M JUKE A LU NDBERG and L. V KER s The effect of DOPA on the spinal cord. I Influenc of t ansmission from pr mary afferents. *A t physiol. and* 1966a, 67 3 3 38
ANDE N E M. G M J and A. LU DBE G The effect of DOPA on the spinal ord. 2. A pharmacological analysis. *A t physiol. sc d* 1 6b 67 387—3 7
APPEL BAD B P BE SOC and Y LAPORTE, Action of t tic and dynamic fusimotor fibres on secondary endings of t pindles *J Physiol* (Lond.) 1 6 185 160—171
B RKER, D *The ac atio f mammalian skeletal m scle* J Ciba Foundation S mposium on Myotatic Kinesthetic and Vestibular mechanisms. J & A. Churchill Ltd London) 1967 3—15.
BERGM J and S GRILLNER Reflex ac n tion and egulation of spontaneo activity in static and d namic γ-motoneurones *B ai R sear h.* 1 67 5 114—11
BESSOU P F EMONET D D nd Y LAPORTE Motor fibres inner ting extrafusal and intrafusal muscle fibres in the cat. *J Physiol* (Lond.) 1 5 180 649—67
BE SOC P and Y LAPORTE, Technique de preparation d'une fib fferent I et d'une fibre aff rent II innervant le même fuse neuromusculaire hez le hat. *J Phy l.* (Paris) 19 57 511 20
BE SOC P and Y LAPORTE Observations on static fusimotor fibres. I *M sc les Afferent d Motor Co t ol.* Nobel Symposium I 1966 81 —8 Ed. R. Granit.
BROWN, M C A CR WE nd P B C M TTH w Obser tions on the f imotor f ers of the tibialis pos erior muscles of the t *J Physiol* (Lond.) 19 5 177 140—1 9
BROWN M C and P B C M TTHEW On he ubdivision of the efferent fib es to muscle pindles into tatic and dynamic fusimotor f bres I *Cont ol nd I n atio Skeletal M scle* Ed B L Andrew D C Thompson & Co Ltd., Dundee Scotland 1 6 18 34
C w A and P B C M TTHEW The effect of timulation of tatic nd dynamic fusimotor fibres on he response to tretching f the primary ending of muscle pindles *J Ph siol.* Lond 1964a. 1 4 100 131
CROWE, A and P B C M TTHEW Further studies of static and dynamic f simotor fibres. *J Physiol* Lond. 1 64b 1 4 132 151
GRILL S T H NGO and A L NDBE G The effec of DOPA on the spinal cord. Reflex activa ion of static γ-motoneurones from the flexor reflex afferents. *Act physiol. scand* 1967 70 403 411
HUNT C C The reflex activity f mammalian mall-nerv fibres. *J Physiol.* (Lond 1951 115 456 46)
HUNT C C., Drug effect on mammalian muscle pindles. *F d Proc* 195 11 5
H NT C C Relation of f nction diameter in afferent fibres of muscle nerves *J gen. Physiol.* 1954 38 117 131
HUNT C C and A S PAI ar Spinal reflex regulation of fusimotor neurones *J Physiol* (Lond.) 1958 143 19 1
J K J K. S and P B C M TTHEW The entral ontrol of the dynamic esponse of muscle spindle eceptors *J Physiol* Lond 19 2 161 357 378.
J J K. S and T R oj ro Fusimotor activity in flexor muscle of the decerebrate cat. *A t physiol sc d* 1 65 63 23 24
LINDSLEY D F and E. ELDRED Spontaneous acti ty in γ-efferents of deafferented spinal cord segment. *P oc Soc p Biol N Y* 1960 103 413 415.
M TTHEW B H C Nerv endings in mammalian muscle *J Physiol.* (Lond 1933 78 1—53
M TTHEWS, P B C The differentiation of two t pes of fusimotor fibre by thei effects on the dynamic response of muscle pindle primary endings. *Quart J p. Physiol* 19 2 47 324—333
SCHÄFER, S S The acceleration response of primary muscle spindle ending ramp stretch of the extrafusal muscle *Experientia* Basel 19 7 23 1026
VOORHOE E P E., Autochthonous activ of fusimotor neurones in the ca *A t physiol. pharmacol. neerl.* 1960. 9 1—43

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From the Institute of Zoophysiology, University of Oslo, Norway

Insulative Changes in the Harp Seal Pup during Moulting

By

Jo Frisch and Nils Are Øritsland

Newborn harp seal pups *(Pagophilus groenlandicus)* are covered in a coat of stiff white wool about 25 mm thick. During the suckling period, approximately 10—12 days, the pups acquire an additional layer of blubber up to 5 cm thick. The pups, now named "white coats" by sealers, refuse nourishment during 2—3 weeks following the suckling period.

As early as two weeks after birth the white coat may be shed to reveal a grey short-haired coat. This moulting process, completed within a few days of its initiation, normally takes place before the animals are 4 weeks old (King 1964, Sivertsen 1941).

Moulting obviously decreases passive peripheral insulation. This decrease may be compensated for by increased heat production and by proper physiological adjustments of the peripheral circulation.

In order to determine the magnitude of decrements in passive insulation temperature gradients throughout the white coat on the dorsal side of a moulting pup were measured. Compensatory shifts in tissue insulation were revealed by means of a heat flow disk which could be placed between the white coat and new grey shorthaired fur without disturbing the animal.

Direct measurements of the insulative value of the fur were obtained to recheck results of measurements obtained on the living pup. The white coat of a moulting pup was attached directly to guarded heat plate using a thin layer of dehydrated lanolin.

Skin temperature measured beneath the white coat of the living pup was found to be 34 ± 1 °C at an ambient temperature of 6 °C. The temperature gradient in the inner most 7 mm of the white coat was 22.6 ± 2° C/cm. Voltage measurements taken with the heat flow disk placed in various positions were

Between the grey and white coat	— 22 microvolts
Top of white coat	— 20 microvolt
Top of grey coat	— 42 microvolts

Conductivity within dense fur as measured in still air is 0.07—0.12 mcal/cm² sec°C (Hammel 1955, Hart 1956, Scholander et al. 1950, Tregear 1965). This corresponds to a heat flow of 158 · 10 to 271 · 10 cal/m²sec. Total insulation

be obtained by dividing the temperature difference across the pelt (31 C—6?= 28 C) by the heat flow. This results in values ranging from 1.8 10⁴ to 1.0 10⁴ cm²sec C/cal or in clo units (Burton 1955) 2.8 to 1.6 clo. Calculated mean insulative value will be 1.4 10 cm²sec C/cal or 2.2 clo corresponding to a conductivity of 0.09 mcal/cm²sec C. The direct measurement on the mounted white coat resulted in an insulative value of 2.4 ± 0.1 clo at ambient air temperature 25 C and heat plate temperature 48.5 C.

Application of the calibration factor for the heat flow disk showed the measured heat flow from a living pup to be more than 100 times greater than the calculated heat flow. This phenomenon is most probably an artifact due to accumulation of thermal energy on the central side of the heat flow disk. We assume however that the thermal energy accumulation rate remained constant throughout measurements i.e. when placed on the white and grey fur. Thus heat flow recordings indicated no significant immediate physiological compensation for the 2.2 clo decrement in insulation during the moulting process.

The experimental animal shed 90 per cent of the white coat within a period of two days. The animals weight was recorded at 19.0 kg. Surface area corresponding to this weight (Irving, Solandt and Fischer 1936) was estimated at 0.08 19.0$^{2/3}$ = 0.57 m Surface area on the experimental animal, excluding limbs and anterior half of the head was measured by dividing the animal into two cones and one cylinder and resulted in an area of 0.44 m². Thus the poorly furred limbs and anterior half of the head represents 23 per cent of the total surface area. If the white pups have developed physiological control of peripheral insulation, a decrease in ambient temperature from 10 C to −10 C calls for a doubling of the oxygen consumption in order to maintain deep body temperature 37 C and skin temperature 34 C.

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References

BURTON, A. C. *M [illegible] old [illegible] ir [illegible] ment* Edward Arnold, London 1955 pp 73
HAMMEL, H. T. Thermal properties of fur. *Amer. J. Physiol.* 1955, *182* 369–376
HART, J. S. Seasonal changes in insulation of fur. *Canad. J. Zool.* 1956, *34* 53–57
IRVING, L., O. M. SOLANDT, D. Y. SOLANDT and K. E. FISCHER. The respiratory metabolism of the seal and its adjustment to diving. *J. [illegible] comp. Physiol.* 1936, *7* 137–151
KING, J. E., *Seals of the world.* British Museum, London 1964 pp. 154
SCHOLANDER, P. F., V. WALTERS, R. HOCK and L. IRVING. Body insulation of some tropical and arctic mammals and birds. *Biol. Bull. mar. biol. Lab.* (Woods Hole) 1950, *99* 225–236.
SIVERTSEN, E., *Biology of the harp seal.* Hvalrådets skrifter No. 26. Oslo 1941 pp 166.
TREGEAR, R. T. Hair density, wind speed and heat loss. *J. appl. Physiol.* 1965, *20* 796–801

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Split Beam Device for Ink Jet Oscillographs

By

JAN GÖTHLIN and TORD OLIN

The term split-beam refers to a special technique for cathode-ray oscilloscopes. With only one electron gun, two occurrences can simultaneously be recorded by an additional device that rapidly switches between two input-amplifiers. Thus, the electron beam for a short moment reacts to the signal of one of the amplifiers and immediately thereafter to the signal of the other. The switching is so fast that it does not disturb the oscillogram.

A similar principle has been adapted by the authors for a direct writing polygraph (Mingograph, Elema). Two preamplified signals are led to a reed relay double pole and double throw (DPDT) Fig. 1. The switches of the reed relay are actuated by a squarewave current through the coil. The two signals are thus alternatingly amplified and recorded by the polygraph. The oscillator generating the switch frequency is an astable multivibrator with two transistors. Fixed frequencies can be set by a system of trim potentiometers and a pushbutton selector. After the oscillator there is an amplifier feeding the coils of the reed relays. The frequency of the output amplifier is a squarewave enabling a rapid, synchronous and [illegible] switching of each relay. The highest frequency possible with the types of relays used is around 80 Hz.

In our original device the switch-frequencies could be set between 1 and 80 Hz. At frequencies above 50 Hz, however overshooting of the ink jets appears together

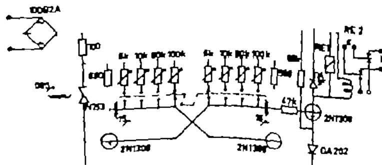


Fig. 1. Circuit for split-beam device. From left to right rectifier oscillator amplifier and reed relays. Two different signals and b, are alternatingly connected with Rela[illegible] 75-10 225, Processor Solna, Sweden.

2 A

being moved with the screws of the cross motion stage under the centre of the hair line cross. The same principle is used for the mapping of structures of interest within the histological section.

The advantages of the described equipment for mapping are evident: a good accuracy, saving of time, a free choice of optic magnification in the microscope and a choice between different magnifications for the transference from the histological section to the diagram.

D 2

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A Possible Difference in the Effect of Chloride on the Surface Membrane and the T-Tubuli Membrane of Hagfish Twitch Muscle

By

N. J. Brautaset and K. Nicolaysen

A sudden reduction of the chloride concentration in extracellular fluid causes a very fast depolarization of frog twitch muscle fibre ($t_{0.5}$<0,5 sec) (see Hodgkin and Horowicz 1959, 1960).

Twitch muscle fibres from the Atlantic hagfish showed a strikingly different behaviour. When {Cl} was suddenly reduced, the membrane was slowly depolarized ($t_{0.5}\simeq$60 sec) (Fig. 1A).

If the same experiment was repeated after destruction of the t-tubuli system of the sarcoplasmatic reticulum by glycerin-treatment (Howell and Jenden 1967, Eisenberg and Gage 1967, Gage and Eisenberg 1967), the depolarization was practically instantaneous (Fig. 1B). Furthermore the potential displacement was much smaller

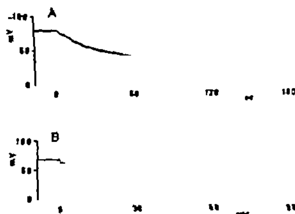


Fig. 1. Change of membrane potential (ordinate) by sudden reduction of $\{Cl\}_o$ from 600 mM to 10 mM at 0 time (abscissa). A. Normal fibre. B. Glycerin-treated fibre.

in such fibres. When {Cl} was reduced from 600 mM to 10 mM the normal fibre was depolarized from −80 mV to −12 mV (Fig 1A) In contrast, the glycerin-treated fibre was depolarized from −70 mV to −61 mV only (Fig 1B)

Tentatively we conclude that the slow change in membrane potential following a reduction of $\{Cl\}_o$ is due to delayed exchange in the t tubuli system. Chlorid seems to have greater effect on the membrane potential when present at the t tubuli membrane than at the surface membrane.

References

EISENBERG, R. A. and P W GAGE, *Scien* 1967 *158* 1700—1701
GAGE, P W and R. S. EISENBERG *Scien* 1967 *158* 1702—1703
HODGKIN A. L and P HOROWICZ, *J Physiol.* (Lond.) 1959 *148*. 127—160.
HODGKIN, A. L. and P HOROWICZ, *J Physiol.* (Lond.) 1960. *153* 370—385.
HOWELL, J N and D J JENDEN *Fed Proc* 1967 26. 553

D 3

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Failure to Influence Autonomic Reflexes by Hippocampal Stimulation in Unanesthetized Rabbits

By

R. S FELDMAN B. R. KAADA and T LANGFELDT

From previous studies t appears unlikely that the hippocampus has direct influence on utonomic activ ties, at least during an anesthetic state. The aim of the present study has been to investigate the possibility (1) that without anesthesia there may be some effects, and (2) that the hippocampus may exert a modulating influence on n inhibitory or excitatory reflex background activity Such was the case when cerebella ffects on tonomic activ ties were investigated by timulation (*Moruzzi, 1950* p. *77—83*)

All tests were done on unanesthetized rabbits To study the effects on an *in hibitory* reflex, olfactory induced respiratory arrest and bradycardia was used With respect to *cit tory* reflexes background activit a conditioned emotional response w th ccelerated respiratory and cardiac activ ty was employed. In these tests the animals were given *isual* timulus 10 secs before a 1 sec electric shock to the hind limb Records were obtained of respiratory movements, pulse rate and the lectrical activ ty of the hippocampus and neocortex.

During timulation of the dorsal or ventral hippocampus thro gh implanted bipolar electrodes, contralateral hippocampal responses were monitored with an oscilloscope to assure that the timulating and recording electrodes were located in the

hippocampus proper and that the timulus parameters produced typical evoked responses on the monitored side

The principal findings were

(1) Hippocampal stim lation alone without the above-mentioned ind ced re flexes, initially prod ced a weak arousal response manifested by neocortical de synchronization, hippocampal 5—6/sec theta activity respiratory and cardiac ac c leration. These effects were absent after 5 to 8 presentations showing rapid habituation.

(2) The olfact ry induced inhibitory reflex. — Tobacco smok blown into the rabbit s nostrils produced an immediate arrest of respiration, a pronounced brady cardia an appearance of hippocampal theta-activity and a neocortical desynchronization. No habituation occurred in this test even when smoke was admini tered as many a 10 t 20 times over a period of 3—4 hrs. In n case concomitant hippocampal stim lation altered the responses to the moke stimulus.

(3) The conditioned autonomic responses —Hippocampal stimulation never altered the respiratory and cardiac acceleration, or the hippocampal theta activity and neocortical desynchronization, recorded as concomitants of arousal and fear in anticipation of foot shock

Even prolonged electrical seizure discharges in the hippocampu did not alter the resting level of respiratic n and pulse rate or the reflexly induced respiratory and cardiac changes.

It is concluded that it is dul ou that the hippocampus exerts a ignificant effect th effect r d of these autonomic functions

References

Mor zi C Problems in erebella physiology C C Thomas, Spr ngfield, Ill 1950 116 pp

D 4

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Obesity Produced by Iron in the Ventromedial Hypothalamic Nucleus of the Rat

By

E D in and H Ursin

It has been known for a long time that lesions in the ventromedial hypothalamic nucleus (VM) produce obesit (weight increase significantly above normal range) in the rat. Reynold (1963 1965) has suggested that the hyperphagia is not due to the lesion of VM but, rather results f om metallic implant in the same

region. The experiment to be reported was an attempt to test this possibility directly. Stereotaxic lesions were introduced by anodal as well as cathodal DC electrolysis through electrodes of ordinary iron, stainless steel and platinum.

The material consisted of 100 rats, 23 controls and 77 brainlesioned animals. Rats were ascribed at random, in a balanced order to the various combinations of electrode type and type of current (1 75 mA, 20 sec) A total of 25 lesioned rats died postoperatively or were excluded for other reasons. Of the remaining 5? brainoperated, 15 were accepted as having a VM lesion affecting at least half the nucleus. Each rat was weighed daily in the first postoperati e week later with longer intervals. They were given dry pellets *ad lib* and limited amounts of milk. The total duration of the experiment was 40 days. The brains were sectioned at 16 μ every ?0th section was stained with thionine adjoining sections were stained with potassium ferrocyanide and counterstained with safranin.

Histological evaluation of th sections was performed without knowledge of changes in body weight. In the potassium ferrocyanide sections, ron implants were clearly evident by well-delimited areas of dark blue stain (Prussian blue) Most iron was found in lesions produced by anodal electrolysis through iron electrodes. Iron deposits were also sometimes found in brains where such implants were not intended. The results showed that the weight increase in the animals w th iron in the VM lesion was significantly greater than in the VM lesioned animals without iron in the lesion ($P<0.002$) and also greater than the sham operated control group ($P<0.002$) There was no significa t difference n weight gain between the two latter groups. Hypothalamic iron implants by itself without a significant lesion, do not produce any obesity ($P=0.6$) In the rats with iron implants obesity is only seen when the VM nucleus is lesioned ($P=0.05$ all P's gi en for two tailed tests)

The results confirm the suggestion from Reynolds (1963 1965) and Rabin and Smith (1968) Hypothalamic obesity is not due to th lesion of VM only it is also necessary that Prussian blue positive substance is implanted. This substance is most probably iron. Any lesion produced by our procedure had definite probability of producing Prussian blue positive substance, probably iron from hemoglobi destruc tion in hemorrhages E en radio-frequency lesions may prod ce iron implants (Herrero 1967)

References

HERRERO, S M Thesis for Ph D Univ Calif Berkeley 1967 Ann Arbor Michigan University Microfilms, N 68-5737 1968
RABIN, B M and C J SMITH, *Physiol. Behav* 1968. *3* 417—420.
REYNOLDS R W *Amer J Physiol* 1963 *204* 60—62
REYNOLDS, R W *Psychol Rev* 1965 *72* 105—116.

COMMUNICATIONS

C 1

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Metabolic Effects of Prostaglandin E_1 in Canine Adipose Tissue in situ

By

B. B. Fredholm and S. Rosell

Recently Shaw and Ramwell (1968) demonstrated that prostaglandins are released from adipose tissue by procedures which also increase the release of free fatty acids (FFA). Since moreover prostaglandins have been reported to be potent inhibitors of FFA formation (see Bergström *et al.* 1968) there is reason to investigate into the physiological role of these substances in adipose tissue. The present study is part of such an investigation.

Subcutaneous adipose tissue from fed female mongrel dogs was isolated and perfused with defibrinated blood at a constant rate (Rosell 1966). PGE_1 was infused in amounts calculated to give a final concentration in blood ranging between 0.4×10^{-9} and 0.6×10^{-6} M.

Over the entire dose range PGE_1 caused an increased uptake of glucose by the adipose tissue, an effect described earlier (Böhle *et al.* 1966). The effects on lipolytic activity were complex, however. Thus FFA and glycerol release showed an initial transient increase following PGE_1 in concentrations between 0.5×10^{-9} and 0.6×10^{-7} M. After the PGE_1 infusion was stopped a second transient increase in lipolysis was observed. This was seen with all doses. Finally signs of a decreased basal lipolytic rate were observed with the highest PGE_1 concentrations.

The ability of PGE_1 to antagonize the increased lipolysis produced by sympathetic nerve stimulation was also studied. The threshold for this inhibition was found to be around 10^{-9} M. About 50% reduction was seen with 0.6×10^{-7} M PGE_1, whereas with a ten times higher concentration the inhibition was virtually complete.

Our results indicate a dual action of PGE_1 on lipolysis. Thus, PGE_1 produces an inhibition of lipolysis, whether stimulated or basal, but also in a certain dose-range a transient increase in lipolysis.

References

Bergström, S., L. A. Carlson and J. R. Weeks, *Pharmacol. Rev.* 1968. 20. 1—48.
Böhle, E., H. Ditschuneit, J. Ammon and R. Döbert, *Verh. dtsch. Ges. inn. Med.* 1966. 72. 465—469.
Rosell, S., *Acta physiol. scand.* 1966. 67. 343.
Shaw, J. E. and P. W. Ramwell, *J. biol. Chem.* 1968. 243. 1498—1503.

C 2

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Reduced Effector Response to Nerve Stimulation in the Cat Spleen after Administration of Prostaglandin E_1

By

P Hedqvist

Experiments were carried out in the isolated perfused cat spleen to study the effects of prostaglandins on the contraction of vascular smooth muscle in response to sympathetic nerve impulses.

Electrical stimulation of the splenic nerves at 10/sec for 20 sec produced a large rise of the perfusion pressure in the isolated spleen. Intraarterial infusion of prostaglandin E_1 (PGE_1) in doses ranging from 0,015 to 1,3 μg/ml of perfusion fluid markedly reduced this response. The inhibitory effect progressively increased with the PGE_1 dose. After cessation of the infusion of PGE_1 the pressor response to nerve stimulation gradually returned to reach the preinfusion level after 20 to 30 min. The pressor response was more efficiently inhibited by PGE_1 at a stimulation frequency of 3/sec than at 10/sec.

Experiments were also carried out to test the effect of PGE_1 on the response to intraarterial injection of noradrenaline (NA). PGE_1 was found to inhibit the pressor response caused by injection of NA as readily as that resulting from nerve stimulation.

PGE_1 has been reported not to affect the mechanical response to nerve stimulation in the dog spleen (Davies and Withrington 1968). The results of the present study however clearly demonstrate that PGE_1 antagonizes the effector response to nerve stimulation and to injection of NA in the isolated, perfused cat spleen.

Prostaglandins of the E series are widely distributed in animal tissues (Bergström and Samuelsson 1965) and are known to be released in response to a variety of stimuli, *e.g.* by stimulation of the splenic nerves in the dog (Davies, Horton and Withrington 1968). Thus it appears conceivable that local release of PGE_1 may play a modulatory role in the vascular sympathetic neuro-effector system.

References

Bergström S and B Samuelsson *Ann. Rev. Biochem* 1963 *34* 101—108.

Davies, B N E W Horton and P G Withrington *Brit J Pharmacol* 1968, *32* 127—135.

Davies, B N and P G Withrington, *Brit J Pharmacol* 1968 32 136—144

C 3

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Interpretation of the Repetitive Impulse Discharge of Motoneurones

By

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C 4

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Nature and Distribution of Inhibition in a Simple Cortex (Dentate Area)

By

T. Lømo

Single perforant path volleys excite the granule cells of the dentate area monosynaptically through synapses located on the middle third part of the apical dendrites. After their discharge the granule cells are subject to postsynaptic inhibition with a maximal duration of about 100 msec (Andersen, Holmqvist and Voorhoeve 1966).

The dentate area is a strictly laminated structure with the granule cell bodies densely packed in one single layer. A strong antidromic or orthodromic volley evoked a compound spike which was maximal in the granule cell body layer. The population spike signalled the near synchronous discharge of a large number of granule cells. It was always followed by a slow wave which was maximally positive in the cell body layer and reversed abruptly to a negative wave in the dendritic region. When precautions were taken to activate the perforant path selectively, the slow wave was always associated with inhibition of a subsequent test spike whether orthodromically or antidromically produced. The slow wave coincided with the first part of intracellularly recorded IPSPs. The exact onset of orthodromically evoked IPSPs was difficult to measure, but occurred from 3—5 msec after the spike discharge. In agreement with this finding the inhibition of a second test spike was never observed earlier than 3 msec after the onset of the conditioning population spike. This relatively long interval may be accounted for by conduction in a recurrent inhibitory pathway and supports the interpretation that the inhibition is primarily, if not exclusively, recurrent.

An antidromic spike actively invaded the granule cell dendrites for at least 2/3 of their total length as shown by the negative component of the antidromic spike. It could be partially suppressed by a preceding ortho- or antidromic volley. When the compound test antidromic spike was reduced to half the control valu no addi tional decrement of the surviving impulses cond cted along the dendrites was observed. This finding and the depth profile of the slow positive wa e indicate that the inhibition is restricted to the dentate granule cell body region.

A single perforant path volley activates the granule cells by means of boutons en passage along a narrow beam orientated approximately parallel to the midline. It was found that the inhibition was distributed primarily to both sides of th activated beam. Maximal inhibition occurred up to 0.5 mm lateral or medial to the excited beam. The positive wave was recorded on-beam" as well as off-beam The peak latency of the positive wave as well as the latency to onset of the associated inhibi tion increased as the recording electrode was moved away at right angles to the beam of activated granule cells. Therefore, the distribution of the inhibition in the dentate area resembles that of the cerebellum.

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References

Andersen, P. B. Holmqvist and P. E. Voorhoeve, *Act physiol scand* 1966. 66 448—460.

C 5

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Circulatory Effects Mediated Through Cardiac Vagal Afferents

By

B. Öberg and S. White

Circulatory responses to electrical stimulation of thin agal afferents serving mainly cardiac receptors (Jarisch and Zotterman 1948) were studied chloralose-anesthetized cats. The rol of these receptors was also studied nder more physiological conditions by observing the effects of ulsion of the nerves on the resting circulation and on th responses to hemorrhage. Blood pressure and heart rate renal, intestinal and skeletal muscle blood flow (enous outflow drop recorders) and changes enous t ne in skeletal muscle nd intestine (plethy mographic technique) were tudied. Buffering nfluences from other receptors were minimized by blockade of the opposite agus and carotid occlusion.

Supramaximal stimulation (8 volts, ? msec) of right or left cardiac afferents produced reflex responses, noticeable even with 2 imp/sec, and maximal with 15 imp/sec characterized by a marked bradycardia, mainl of reflex vagal origin, blood pressure fall and reflex inhibition of sympathetic tone in all studied vascular beds. This response pattern involved a more extensive reflex bradycardia and reduction of kidney flow resistance for a given reduction of muscle flow resistance than the reflex responses to arterial baroreceptor stimulation (tugging of the carotids) In a few experiment the reflex bradycardia was accompanied by an increased vasoconstrictor tone and blood pressure rise

Avulsion of the vagal filaments did not in itself produce any marked effects on resting circulation, irrespective of the degree of filling of the central veins or pre ailing sympathetic tone neither were the circulatory responses to a moderate hemorrhage significantl influenced However rapid withdrawal of blood from a catheter in the right atrium often resulted in a marked bradycardia, which was abolished by a ulsion of the cardiac nerves.

The data suggest that the bursts of activity known to occur in cardiac afferents, has only small effects on the neural control of the circulation. The pronounced cardiac responses to even low-frequency electrical stimulation of the nerve therefore seem to imply activation of afferent pathways not normally during rest or moderate hemorrhage The similarit of responses, obtained with electrical stimulation and with rapid bleedings from central blood reservoirs, might indicate that the fibres are gaged in an emergency reflex, initiated by exceedingly poor filling of the heart which acts mainly to "break the heart and possibly inhibit peripheral sympathetic tone.

This study was supported by grants from the Swedish Medical Research Council, from Air Forc Office of Scientific Research, OAR, United States Air Force under Contract F6 103 -68-C-0014 and from U. S. Public Health Service HE-05675-07)

References

Jarisch A and Y Zotterman Y. *Act physiol scand* 1948, *16* 31—51

C 6

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On Pulmonar Gas Exchange and Regional Acid Base Conditions during Submersion

By

G Clausen and A. Ersland

C 7

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The Effect of Adrenalectomy on Activity of ($Na^{+}+K^{+}$) ATPase in Outer Cortex, Inner Cortex and Medulla of the Rat Kidney

By

P. L. JØRGENSEN

In the studies hitherto reported on the regulation of ($Na^{+}+K^{+}$) ATPase in rat kidney (Landon, Jazab and Forte 1966, Chignell and Titus 1966 Jorgensen 1968) activity has been measured in homogenates and subcellular fractions prepared from whole rat kidneys. Here an attempt is made to localize the changes in activity of ($Na^{+}+K^{+}$) ATPase observed after adrenalectomy to definite parts of the nephron by measuring the quantitative distribution of the enzyme in the four zones identifiable by naked eye examination of transverse sections of the rat kidney (McFarlane 1941)

Tissue from each of the four zones was obtained by dissection of transverse sections of the kidney The tissue was homogenized and centrifuged at 260,000×g for 60 min. The supernatant had not ATPase activity but contained 40 % of the total protein in the homogenate The pellet containing all particulate structures in the homogenate was resuspended by homogenization. The ouabain sensitive, ($Na^{+}+K^{+}$) dependent ATPase and glucose-6-phosphatase activity was measured after preincubation with deoxycholate and EDTA (Jorgensen 1968)

In accordance with the histochemical localization of glucose-6-phosphatase to the proximal tubules (Wachstein and Meisel 1957) activity of this enzyme was high in the outer cortex (28.3 ± 1 1 μM Pi/mg protein/hr) low in the inner cortex (8.5 ± 0 8) and zero in the medulla and the papilla.

In the medulla, where the predominant histological feature is the broad limbs of Henle, activity of ($Na^{+}+K^{+}$) ATPase was strikingly high (Table I) while activity in the grey papilla, containing the thin limbs of Henle and collecting ducts, was con-

TABLE I Activity of ($Na^{+}+K^{+}$)ATPase in homogenates prepared from subdivisions of kidney from normal and adrenalectomized rats Mean values ± S.E.M. are given

	Normal rats (n=8)	Adx rats (n=9)	change	p
	μM per mg protein per hour			
Outer cortex	35.8 ± 0,9	27 1 ± 1 4	—24	< 0,001
Inner cortex	42.3 ± 2,7	24 9 ± 1 3	—41	< 0,001
Medulla	83.1 ± 3,5	49,8 ± 3,8	—42	< 0,001
Papilla	11 1 ± 1,2	—		

siderably lower than in the other zones. In agreement with this finding on homogenates activity in the heavy microsomal fraction prepared from the medulla was 170—220 μM Pi/mg protein/hr and 3 fold higher than the ouabain sensitive activity in the microsomal fraction prepared from whole kidneys. These large differences in activity underline the necessity of measuring the activity in subdivisions or individual tubules of the kidney.

8 days after adrenalectomy a considerable reduction in activity of (Na + K) ATPase was found in the inner cortex and medulla, which is dominated by distal parts of the nephron. In the outer cortex, where the proximal tubules predominate the decrease in activity was smaller.

These results suggest a localization to the distal parts of the nephron of the decrease after adrenalectomy in activity of (Na + K) ATPase in rat kidney.

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References

LANDON, E. J., N. JAZAB and L. FORTE, *Amer. J. Physiol.* 1966. *211*. 1050—1056.
CHIGNELL, C. F. and E. TITUS, *J. biol. Chem.* 1966. *241*. 5083—5089.
JØRGENSEN, P. L., *Biochim. biophys. Acta* (Amst.) 1968. *151*. 212—224.
MCFARLAND, D., *J. Path.* 1911. *5*. 17—24.
WACHSTEIN, M. and E. MEISEL, *J. Histochem. Cytochem.* 1957. *5*. 201—220.

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Some Effects of Exclusion of Macromolecules from Fibrin

By
U. SÖDERBERG

In a study of methods of measuring free fatty acids (FFA) in blood we found to our surprise that the concentration is higher in serum than in plasma. Similar results were obtained when the FFA concentration was measured in citrate plasma before and after coagulation with thrombin and in model systems made of mixtures of human serum albumin and fibrinogen. It was concluded that the FFA albumin complex is excluded from the fibrin. The excluded volume was found to be at least 20 times the volume expected from the dry weight of fibrin (Söderberg and Söderberg 1967).

Further experiments on radioactive thyroxine in plasma and serum have confirmed the previous results on FFA. In order to improve resolution of the measurements attempts were then made to study these effects with gel filtration on fibrin columns. Laurent and Killander (1964) suggested that dextran gels allow filtration of molecules according to their size as a consequence of steric exclusion of macromolecules

from a three-dimensional network of rods, and Laurent and co-workers have studied the properties of connective tissue polysaccharides successfully with gel filtration (Laurent 1968 for references)

In our experiments, bovine and human fibrin were used in columns and separation of iodide albumin and trypsin were studied. Radioactive tracers facilitated identification so as to allow the use of small columns. The substances tested could easily be separated, but there was both true gel filtration and effects of charge interactions. Changes in pH and ionic strength of the eluant had therefore to be employed. The results show that the exclusion volume for albumin was comparable to that of the previous batch experiments. The distribution of ions thus differs from that of macromolecules in plasma containing a fibrin clot. Trypsin was not excluded from the whole albumin-free space. Whether this depends on the small size of trypsin or on interaction with fibrin is not clear since the addition of albumin to the solvent could force trypsin to follow albumin within the void volume.

In preliminary experiments also plasmin (fibrinolysin) was studied on the fibrin columns. At low pH a peak of plasmin appeared in the void volume, a tail followed and there was low recovery

The findings are believed to be of importance for the understanding of mechanisms of fibrinolysis, and, if coagulation processes interfere with transport in the walls of the blood vessels, also of exchange of substances between blood and tissues. They are also support of the biological implication of exclusion phenomena presented in the literature (Laurent 1968)

References

LAURENT T C., I The Chemical Physiology of Mucopolysaccharides. Ed. G Quintarelli, Little, Brown and Co Boston 1968 pp 153—170

LAURENT T C and J KILLANDER, J Chromatogr 1964 14 317

SÖDERBERG, B and U SÖDERBERG, Lif Sci 1967 6 1015—1021

C 9

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The Rate of Respiration and C —C_6 Ratio in Glucose Oxidation in the Rat Gastrointestinal Tract

By

A. AITIO and O HÄNNINEN

It is known that the oxygen consumption of the small intestine of the cat decreases from the oral to the aboral end (Dorman and Steggerda 1961) and that the rate of hexosemonophosphate oxidation is very high in the intestinal mucosa of the rat (Enterman and Kay 1959) The purpose of the present study was to compare the

TABLE I Th oxygen pt k ca bon dioxid production, respiratory quotient (R.Q.) nd C_1—C_6 ratio during glucose oxidation l whole wall slices from the rat gastrointestinal tract. The mber of th determinations in parentheses. P alues are given.

		O_2 uptak µl/hr.mg of dry weight	CO_2 production	R.Q.	C_1/C_6
Glandular stomach	I	22 4 ± 1 4 (32) I—II P 0.001	18.2 (10)	0.81 ± 0 08	1 41 0 08 (12) I—II P < 0 2
Duoden m	II	14 6 ± 0 6 (34) II—III P 0.001	11 4 (8)	0.81 ± 0 05	1.69 ± 0.16 (19) I—III P 0 00
Jejun m	III	8.8 0.6 (32 III—IV P 0 05	7 6 (10)	0 87 ± 0 03	1 84 0.15 (15) I—IV P 0 005
Terminal ileum	IV	6 3 ± 0.4 (27) IV—V P 0 005			2 42 ± 0.18 (17) I—V P 0.001
Cecum	V	4 6 ± 0.3 (27)	4 1 (9)	0 90 ± 0.04	2.34 ± 0.18 (17) I—VI P 0 00
olon	VI	5.2 ± 0 3 26	3 9 (11)	0.75 ± 0 05	2 17 ± 0.22 (16)

rat oxygen uptake carbon dioxide production and C —C_6 ratio in glucose oxidation of various segment of the rat gastrointestinal tract.

Whole wall pecimen of the glandular st mach duoden m, jejunum, terminal ileum, cecum and colon were cut in 0 5 mm lices in a Mickle Laboratory Engineering microtome The slices were incubated in Warburg flasks in glucose-fortified Krebs-Ringer phosphat buffer in an O_2-atmosphere at 37 for up to 1 hr under constant shaking The respir tion was followed by the d'rect Warburg method. For the C_1—C_6 ratio determinat ons th $C^{14}O_2$ pr duced from C_1 and C_6 of the correspondingly labeled D-glucose was bsorbed n 0 3 ml of ethanolamine in the central well. This solution together w th methanol washings (3 × 0.4 ml) was transferred to a counting vessel and 5 ml of scintillation liquid (4 g of 2,5-diphenyloxazol and 0.1 g of 1 4 bis(2 (4-methyl-5-phenyloxazolyl)) benzene in 1000 ml of toluene) wa added. The counting was carried out in a Beckman Mod 1 1650 Liquid Sci tillation System. The counting efficiency was 87 per cent

The results are collected into Table I The oxygen uptake and carbon dioxide production were found to decrease rapidl from the oral to the aboral end of the gut whereas the C_1—C_6 ratio increased from 1 4 in the glandular stomach to 2.2 in the colon. No significant changes were found in t R.Q. alues.

References

Dorman, H. L. and F. R. Strooterda, *Amer. J. Physiol.* 1961 *201* 292.
Exterman, C. and R. E. Kay *J. biol. Chem.* 1959 *234* 1634

C 10

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Biochemical and Morphological Alterations in Livers of Rats Fed High Alcohol and High Fat Diets with Ample Protein and Vitamin Supply

By

J. Ahlqvist, M. Nyholm, M. Salaspuro and H. Wallgren

In view of the uncertainty concerning the role of nutritional factors in the pathogenesis of fatty degeneration and structural alterations of the liver in connection with heavy alcohol intake, the effect was tested of altering the composition of calorigenic nutrients in diets containing ample quantities of protein and vitamins. Liquid diets designed as (A) *high fat-high alcohol* (B) *high fat-high sucrose* (C) *extremely high fat* and (D) *high sucrose-high alcohol* were given as sole food to groups of 5 rats for 82 days. Ingestion of the high fat-high alcohol diet raised the total fat of the liver by a factor of about 2.5 decreased the cytoplasmic redox potential as measured *in vitro* led to appearance of numerous Mallory bodies, and caused marked decrease of liver glycogen. Electron microscopy revealed vesiculation of the endoplasmatic reticulum and marked swelling and degeneration of the mitochondria. Fibrotic alterations and formation of fatty cysts, characteristic of choline deficiency were minute. None of the other regimens induced any clear deviations from normal patterns of liver biochemistry and morphology In contrast to the situation in choline-deficient livers, ethanol caused a marked elevation of the lactate/pyruvate ratio in incubated *slices from the livers of rats on all regimens. No clear difference in rate of elimination* of alcohol *in vivo* was seen It is concluded that the fatty metamorphosis and morphological alterations do not resemble those caused by choline deficiency that alcohol *per se* does not seem to be responsible for the alterations, and that the feeding of large amounts of alcohol and fat greatly promotes the appearance of lesions.

TABLE I Th oxygen ptak ca bon dioxid production, respiratory quotient (R.Q.) and C_1—C_6 ratio during glucose oxidation I whole w ll lices from the rat gastrointestinal tract. The umber of he determinations I parentheses. P alues are given.

		O upt k µl hr.mg of dry weight	CO production	R.Q	C_1/C_6
Glandula stomach	I	22.4 1 4 32) I—II P 0.001	18.2 (10)	0 81 ± 0 08	1 41 ± 0 08 (12) I—II P < 0 2
Duoden m	II	14 6 ± 0.6 (34) II—III P 0.001	11 4 (8)	0 81 ± 0.05	1 69 ± 0.16 (15) I—III P < 0.002
Jejunum	III	8.8 0 6 32 III—IV P 0 05	7.6 (10)	0.87 ± 0.03	1.81 ± 0 15 (15) I—IV P < 0.005
Terminal ileum	IV	6.5 0 4 27) IV—V P 0.005			2.4 ± 0.18 (17) I—V P 0.001
Cecum	V	4 6 ± 0 3 (27)	4 1 (9)	0.90 ± 0.04	2.34 ± 0.18 (17) I—VI P 0.005
lon	VI	5 2 ± 0.3 26	3.9 (11)	0.73 ± 0.05	2 17 ± 0 22 (16)

vitro oxygen uptake carbon dioxide production and C_1—C_6 ratio in glucose oxidation of arous segment of the rat gastrointestinal tract.

Whole wall specimens of the gland lar stomach duodenum, jejunum, terminal ileum, cecum and colon were cut in 0.5 mm slices in a Mickle Laboratory Engineering microtome Th slices were incubated in Warburg flasks in glucose-fortified Krebs-Ringer phosphate buffer in an O_2-atmosphere at 37 for up to 1 hr under constant shaking Th respiration was followed by the direct Warburg method For the C_1—C_6 ratio determinations the $C^{14}O$ produced from C and C_6 of the correspondingly labeled D-glucose was absorbed in 0.3 ml of ethanolamine in the central well This solution together with methanol washings (3×0.4 ml) was transferred to a counting vessel and 5 ml of scintillation liq id (4 g of 2,5-d'phenyloxazol nd 0.1 g of 1 4-bis(2 (4 methyl-5-phenyloxazolyl))benzene n 1000 ml of toluene) was added. The counting was carried out in a Beckman Model 1650 Liquid Scintillation System. The counting efficiency was 87 per cent.

The results are collected into T ble I The oxygen uptake and carbon dioxide production were found to decrease rapidly from the oral to the aboral end of the gut whereas the C —C_6 ratio increased from 1 4 in the glandular stomach to 2.2 in the colon No significant changes were found in the R.Q values.

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Distribution of Recurrent Inhibition of Ia IPSPs in Motoneurones

By

H. HULTBORN E. JANKOWSKA and S. LINDSTRÖM

Impulses in motoneurone axon collaterals inhibit interneuronal transmission in the disynaptic Ia inhibitory pathway to motoneurones (Hultborn *et al* 1968 b). Interneurones with the expected convergence of monosynaptic Ia excitation and recurrent inhibition have been found (Hultborn *et al.* 1968 c)

In the present investigation we have examined the distribution of recurrent inhibition *of Ia IPSPs* in motoneurones belonging to different hindlimb muscles. Cats were anemically decorticated and usually under light Nembutal anesthesia but in a few cases unanesthetized. A few experiments were made on spinal cats. Dorsal roots L_5—S were transected, while all ventral roots were left intact. The motoneurones were antidromically identified and the test was evoked by very weak stimulation of dorsal roots from which reciprocal Ia inhibition would be expected. The distribution of the recurrent effects was obtained from measurements of the depression of the Ia IPSP following antidromic stimulation of the different peripheral nerves. The intracellular records were fed to an average computer (CAT 1000) and the amplitudes of 20 averaged alternating test and conditioned responses were compared.

In motoneurones belonging to the hip flexor sartorius, recurrent conditioning most effective from the hip extensors, adductor femoris, semimembranosus and anterior biceps. The *knee extensor quadriceps*, gives a regular but smaller decrease. In posterior biceps-semitendinosus motoneurones the test is most strongly inhibited from quadriceps, but semimembranosus, adductor femoris and sartorius also have some effect. Ankle extensors and anterior biceps are most potent in pretibial flexor motoneurones while there is hardly any decrease from other hip extensors. The recurrent inhibition seems to be less pronounced in the Ia inhibitory pathway to extensor motoneurones, quadriceps being an exception. In quadriceps motoneurones the test is decreased from the hamstring group and, though less effectively, also from the ankle extensors and pretibial flexors. In semimembranosus-anterior biceps motoneurones the depression is most pronounced from quadriceps but sartorius also gives some effect.

The recurrent effect on the Ia inhibitory pathway can release motoneurones from the reciprocal inhibitory control thus limiting the effect of impulses in Ia afferent to excitation. Although the *distribution pattern is wider than that of reciprocal Ia inhibition* its main feature is the regular and strong effects from efferent to muscles whose Ia afferents give Ia IPSPs in the motoneurones recorded from (cf. Eccles and

Lundberg 1938) This distribution suggests that the functional significance is to allow movements or postures involving a co-contraction of flexors and extensors operating at the same joint.

References

Eccles R. M and A Lundberg J Physiol (Lond.) 1958, 144 71—298.
Hultborn, H E. J kowski nd S. Lindstr Ök Lif Sci 1968 a. 7 337—339
Hultborn H E. J kowski and S. Lindström Act physiol. s a d 1968 b. 73 41 A
Hultborn H., E. J kowski and S. Lindstr Ök Brai R ar h 1968 9 36 —369

C 13

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Nervous and Pharmacological Influences on Fat Metabolism in Mesenteric Adipose Tissue

By

K. Ballard and S Rosell

Modifications in th rate of release of glycerol and free fatty acids (FFA) have been shown to occur in response to several stimuli in perfused subcutaneous ad pose tissue of dogs (Rosell 1966) In the studies reported here important differences in response o some of the same stimuli were found in adipose tissue of the mesentery An isolated of mesenteric adipose tissue was perfused, in situ at a constant flow rate with fibrinated blood. Venou samples were collected t intervals while the preparation was under the influence of sympathetic nerve timulation, 5-hydroxytryptamine (5-HT) histamine compound 48/80 or noradrenaline. The plasma was subjected to analysis for glycerol (Laurell and Tibbling 1966) and FFA levels (Trout et al. 1960)

Nerve stimulation before and after α-receptor blockade with dihydroergotamine, and 5-HT (4 μg—100 μg) failed to evoke any changes in the net release of glycerol and FFA. Compound 48/80 (20 μg—100 μg) was effective in only one out of 13 trials. Histamine in doses from 8 μg to 100 μg consistently increased the release of these compounds into plasma Noradrenaline (2 μg—100 μg) also caused changes in plasma content of glycerol and FFA although the effect was less consistent

These results s ggest that the sympathetic outflow to mesenteric adipose tissue may have a less significant role as a regulating mechanism for lipid metabolism as compared to subcutaneous adipose tissue subjected to similar conditions. Stimulation of sympathetic nerves also appeared to have comparatively less influence on blood flow in the mesenteric adipose tissue than on the segment of intestine attached to it.

Histamine, 5-HT and noradrenaline had effects on mesenteric adipose tissue similar to those seen in subcutaneous tissue Compound 48/80, however appeared to be a much less potent stimulus to mesenteric tissue

References

Laurell, S. and G. Tibbling, *Clin. chim. Acta* 1966. *13* 319—322.
Rössner, S. *Acta physiol. scand.* 1966. *67* 343—351.
Trout, D. L., H. Estes and S. J. Friedberg, *J. Lipid Res.* 1960. *1* 199—202.

C 14

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Synergistic Action of Kallikrein and Phospholipase A on Histamine Release

By

E. Amundsen, P.-O. Hagen and E. Ofstad

C 15

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Measurement of Local Metabolic Rate in Kidney and Brain

By

K. Aukland, S. Flood, J. Johannessen and F. Kiil

In most organs, practically all expended energy is converted to heat within the tissue. Measurement of local heat generation will therefore give a good estimate of local metabolic rate. From internal organs, metabolic heat is removed almost exclusively by blood flow. If blood flow is suddenly stopped, heat will therefore accumulate in the tissue at a rate determined by the metabolism (Janssen and Grupp 1957, Rennie 1965). The rate of heat accumulation, $\dot{Q}$ (cal/min · cm³), can be calculated from the rate of temperature rise: $\dot{Q} = \Delta\dot{T} \cdot C \cdot D$, where $\Delta\dot{T}$ is the rate of temperature increase (°C/min), C = thermal capacity (cal/°C · g) and D = density of the tissue (g/cm³). The initial $\dot{Q}$ is a measure of local metabolic rate provided 1) unchanged tissue metabolism and volume during the first seconds of circulatory arrest, and 2) heat removal only by blood flow.

Temperatures were measured at different sites in the dog kidney by fine thermocouples, and circulation was suddenly arrested by a nylon snare around the renal artery. The rate of heat accumulation in the cortex was practically constant in the

first 3—4 sec, and then fell exponentially The initial Q averaged 0.47 cal/min cm³ corresponding well to literature data on renal oxygen consumption (0.08—0.12 ml/min g Similar values were obtained in the outer medulla, whereas circulatory arrest caused no consistent temperature rise in the inner medulla, probably because heat removal from this zone is mainly dependent on heat diffusion (Aukland 1967) Metabolic rate in the outer medulla was reduced by mersalyl (—50 %) furosemide (—70 %) and ethacrynic acid (—80 %) with much smaller changes in cortical Q suggesting a main effect of these diuretics in the loops of Henle. Chlorothiazide reduced cortical (—20) but not medullary metabolic rate indicating an effect in the convoluted tubules

Measurements in the brain were made in Nembutal anesthetized goats in which the vertebral arteries had been ligated. Circulatory arrest was produced by inflating a cuff around the neck and or clamping the carotids. Preliminary experiments showed a metabolic rate of 0.15—0.20 cal/min cm³ in the cortex and 0.07—0.05 in white matter The absolute values may be too low because of continued circulation through nonoccludable collaterals close to the spinal column, but are comparable to the mean cerebral oxygen consumption measured in Thiopental anesthetized humans (0.021 ml/min g ≈ 0.11 cal/min cm³ Sokoloff 1960)

References

Aukland K. *Circulat Res* 1967 20 194—203

J ... ex S and G G ... *S kmiedebe g* ... *Path Pharmak.* ... 45—256

Reeve, D W *Physiologist* 1965 8 258

Sokoloff L. In Handbook of Physiology Sec 1 Neurophysiology Vol. III Ed. by Field. J H W Magoun and V E Hall Washington D C Amer Physiol Soc 1960 1843—1864

C 16

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Uptake of Kr from the Lumen of the Small Intestine to the Intestinal Blood in the Cat

B

O Lundgren and J Svanvik

In recent experiments on the small intestine of the cat mucosal, submucosal and muscularis blood flows were estimated separately and evidence was presented of a countercurrent exchange in the villi Lundgren 1967 In the present study the transfer of Kr^{85} from the intestinal lumen to the blood was investigated both during rest and during identical reductions of intestinal blood flow brought about alternatively by vasoconstrictor fibre stimulation and graded arterial inflow pressure reductions.

The lumen of a denervated jejunal section was perfused in situ with bodywarm Tyrode solution containing a constant Kr^{85} concentration. The jejunal venous effluent (Q) was continously measured by a drop recorder unit and the relation between the Kr^{85} concentration in this effluent and that in the lumen perfusate was calculated from the activity of concomitantly taken samples. On the basis of the partition coefficient for Kr^{85} water/blood (Lassen and Munck 1955) the amount of the intestinal blood stream (Q_x) which was fully equilibrated with the luminal contents could be deduced. The relation between this, which expresses the Kr^{85} transfer "effective" blood flow and the total intestinal blood flow (Q_x/Q) reflects the efficiency with which easily diffusable lipid soluble substances enter the intestinal blood stream from the lumen.

At rest Q_x/Q was as low as 0.134 ± 0.006 (SE) for Kr^{85} which should be contrasted to the fact that the fraction of intestinal blood flow diverted to the mucosa is of the order of 0.6—0.8 (Lundgren 1967). This low extent of equilibration despite the presence of a huge surface, short diffusion distance and a highly diffusible indicator substance may suggest a trapping of Kr^{85} in the mucosal countercurrent mechanism with a delayed entrance into the venous effluent.

In the steady state phase of low frequency constrictor fibre stimulation which decreased Q about 35 %, Q_x/Q increased significantly from 0.129 to 0.150 ($n = 9$, $p < 0.005$). When similar reductions of Q were brought about by mechanical influence with the arterial inflow Q_x/Q was not significantly altered (0.138 and 0.140, $n = 9$). These results suggest that a neurogenic reduction of intestinal blood flow, which particularly seems to affect the mucosal supply (Wallentin 1967), will cause a greater proportional reduction in blood flow than in the transfer of easily diffusible substances from the lumen to the blood stream. This, in turn, suggests such changes in the microcirculation as to reduce the efficiency of the countercurrent trapping mechanism.

References

Lassen, N. A. and O. Munck, *Acta physiol. scand.* 1955 33 30—49
Lundgren, O. *Acta physiol. scand.* 1967 Suppl. 303
Wallentin, L., *Acta physiol. scand.* 1967 69 Suppl. 279

C 17

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Concentration Profiles for Gas Diffusion in Skeletal Muscle in vitro and in vivo

By

P Sejrsen and K. H Tønnesen

The purpose of the present study was to investigate the effect of blood flow on the progression of a concentration profile of gas in muscle tissue Other experiments (Sejrsen 1967 Sejrsen and Tonnesen 1968) have suggested that vessels greater than capillaries serve as exchange vessels for gases.

A constant tension of Xenon-133 gas was applied to the surface of a cut muscle piece at 37 C. After 1 to 3 min the muscle tissue was frozen instantaneously in iso-pentane precooled by liquid nitrogen In a cryostat microtom (−20° C) 100 μ thick slices were cut from the surface and transferred to small precooled vials, which were sealed immediately It was controlled that only few per cent of the Xenon 133 gas escaped from the slices during this procedure Xenon-133 activity in each sample was counted in a well type scintillation crystal. Similar experiment were carried out on muscle tissue in vivo

The concentration profile in the in vitro studies agreed with that obtained by the k diffusion equation. However the concentration profile in the in vivo study was different. Furthermore the Xenon-133 gas could be demonstrated the double distance from the surface as in the in vitro study These results suggest convection of Xenon-133 with the flowing blood Therefore the blood within vessels greater than capillaries exchanges gas with the tissue In addition the diffusion coefficient for Xenon-133 was determined from the results of the in vitro study B this transient method the diffusion coefficient for Xenon-133 at 37 C was found to be 0 6 10 cm²/sec. Corrected for temperature and molecular weight this figure is in agreement with that obtained by Krogh (1919) for oxygen in skeletal muscle

References

Krogh A., *J Physiol* (Lond 1919 *52* 391—408
Sejrsen, P *Circulat Res* 1967 *21* 281 –295
Sejrsen, P and K. H. T nnesen *Circulat R* 1968, *22* 679—695

C 18

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Effect of Training on Muscular Blood Flow during Exercise

By

J P CLAUSEN and J TRAP-JENSEN

7 patients with coronary artery disease were investigated before and after a 6—8 weeks training program. Ventilation, oxygen uptake, cardiac output and intraarterial blood pressure were measured during exercise on the same work load two times before and two times after the training period. A reduction of ventilation and heart rate was observed. Oxygen uptake was unchanged. The stroke volume increased in all cases but one. Cardiac output decreased significantly in two cases and increased in one, but the average value for the series was unchanged. In two of the patients blood flow in m. vastus lat. was measured during exercise using the ^{133}Xe-clearance technique and a significant decrease (21 %) was observed.

Several investigators have demonstrated that physical training results in a more hypokinetic circulation and explained this by an altered distribution of the cardiac output favouring the working muscles and causing a more effective reduction of the flow in non-active tissues. This explanation disagrees with the finding that the reduction of the splanchnic flow is less pronounced during exercise at the same work load after training indicating that the general sympathetic vasoconstriction activity is reduced.

The findings in our study suggest that the reduction in cardiac output is due to a decrease in the perfusion of the working muscles. Since the total oxygen uptake was unchanged this implies an increased muscular oxygen extraction. When it is considered that red muscle fibers—due to numerous mitochondria and a rich blood supply—have greater capacity for aerobic metabolism than white muscle fibers, an explanation could be, that after training a greater proportion of the work load is performed by means of red muscle fibers.

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Department of Physiology University of Turk Finland

Spontaneous Spindle Activity as a Gating Mechanism for Recruiting Responses in an Unanesthetized Cat

By

I Laitinen and P Valleala

The present study made in unanesthetized cats shows that the spindle activity of natural sleep is a prerequisite for recruiting responses (RRs) in contrast to the finding of Dempsey and Morison (1942) in anesthetized cats that recruiting responses cannot be induced during maximal spindle activity and vice versa"

Stimulation of "non-specific" thalamus at the rates of 6—12/sec elicits RRs in

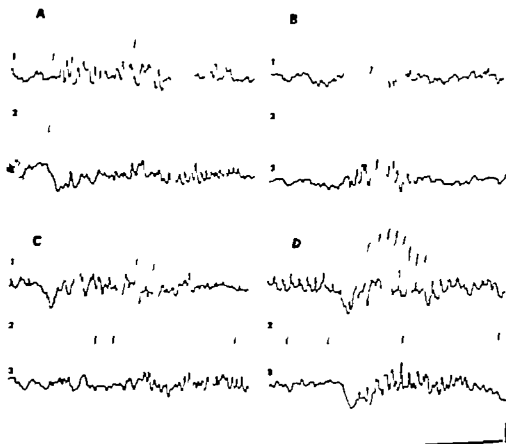


Fig. 1 Repetitive stimulation of nonspecific thalamus during the spindle stage of natural sleep. ECoG left anterior sigmoid gyrus 1 right anterior sigmoid gyrus (3) Stimulation parameters A 1/sec, 0.8 mA B 2/sec 0.5 mA C 4/sec 0.5 mA D 8/sec 0.5 mA. Pulse duration 1 msec. Stimulation artifact (2) Calibrations 0.1 mV 1 sec.

wide cortical areas. The latency of potentials forming RRs is similar to that of component II described by Pollen *et al* (1964)

In the course of long term stimulation performed during the spindle stage of natural sleep RRs but no spindles were visible in the record, providing that the stimulation intensity was high enough to elicit RRs. When using lower stimulation frequencies *e.g.* 1—3/sec, a well-developed component II appeared occasionally during the course of sleep spindles whereas no responses could usually be seen during interspindle periods at the stimulation intensity liminal for RRs (Fig. 1) The amplitude of component II depended on its temporal relationship to a spindle wave as could be expected from the study concerning the events in thalamus during spindle activity (Andersen and Sears 1964) Facilitation i. increase of the amplitude of component II seemed to be more prominent in proreus and anterior sigmoid gyri on stimulated side than in posterior sigmoid gyrus or in corresponding localizations on contralateral side.

Aided by a grant from Juselius Foundation (Finland)

References

Andersen P and T. A. Sears, *J Physiol.* (Lond.) 1964 *173* 459—480.
Dempsey E. W and R. S. Morison *Amer J Physiol* 1942. *135* 301—308
Pollen D A., K. H. Reid and P Perot *Electroenc. clin. Neurophysiol.* 1964 *17* 57—67

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Cortical Projections of Large Muscle Afferents from the Cat's Forelimb

By

H. Silfvenius

Large muscle afferents from the contralateral forelimb project to the region of the posterucíate dimple (Pcd) Oscarsson and Rosén 1963 and to the lower bank of the anterior suprasylvian sulcus (Ssa) Landgren, Silfvenius and Wolsk 1967 Evidence of another primary projection area in the ansate region will be presented in this report, as well as evidence of projection area in the lateral sigmoid gyrus.

In cats anesthetized with chloralose graded electrical stimulation of forelimb muscle nerves was performed. Cortical surface potentials were recorded Depth recordings were made with microelectrodes.

Group I afferents of the distal deep radial-(DDR) the extensor carpi radialis-(ECR) the biceps-(Bic) and the triceps nerves (Tric) project to the rostral bank of the contralateral ansate sulcus. The extent of this locus is approximately 2—3 mm.

The response appears 300 μ beneath the surface of the hemisphere It is evoked at threshold strength of the afferent volley Its amplitude maximum is reached well below 2 T The latency is as short as that of the Ped response (5 msec)

Group I projections were furthermore observed in the lateral sigmoid gyrus, rostral to and separated from the Ped area. DDR, ECR, Bic and Tric evoked responses at 1 T which reached their amplitude maxima below 2 T The latencies of the potentials were occasionally as short as those in the Ped. but generally they were about 0.5 msec longer

Removal of the Ped-area and of the lower bank of the Ssa did not abolish the ansate Group I response Removal of the Ped-cortex reduced the lateral sigmoid response considerably After sectioning of the dorsal columns at C_1 the response disappeared in both loci.

The ansate locus which is located in the cytoarchitectonic area 2 could be related functionally to the sensory cortex or possibly to the nearby association cortex of Thompson Johnson and Hoopes 1963 in areas 5 and 7

The lateral sigmoid Group I area is located in the motor cortex of 4 γ The observed longer latencies and its dependence on the Ped-locus may suggest that this area is a secondary Group I projection, a finding which is compatible with the anatomical observations of Jones and Powell 1968.

References

Jones, E G and T P S Powell, *Brain Res.* 1968. *9* 71—94

S H Silfvenius and D Wolk, *J Physiol* (Lond.) 1967 *191* 543—559.

Landgren O and I Rosén *J Physiol.* (Lond.) 1963 *169* 924—945.

Thompson R F R H Johnson and J J Hoopes, *J Neurophysiol.* 1963 *26.* 343—364

C 21

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Alcohol and Auditory Evoked Responses in Man

By

H Fruhstorfer and P Soveri

The effect of ethanol on the human CNS is reflected in changes of both evoked and spontaneous electrical activity (Davis *et al* 1941 Holmberg and Mårtens 1955 Gross *et al* 1966) The purpose of this study is to analyze the effect of ethanol on the unspecific auditory evoked response in different states of attention and habituation.

Eight healthy subjects participated in the experiments. During a session the subject was stimulated with 40 groups of seven identical clicks (1 group/100 sec 1 click/1 sec 60 dB above threshold) The subject was asked either to expect a stimulus group as attentively as possible or to ignore it by reading The responses recorded from the

ertex were summated selectively in a LINC-Computer. The result represented the seven average responses to a stimulus group. Furthermore, the responses to the first stimulus of a group were summated selectively on the criterion whether the subject estimated his state as attentive or not. In 16 out of 36 expts. the subject received on empty stomach 0.52 g/kg of ethanol diluted in an equal volume of destilled water 45 min before the session (estimated blood alcohol peak 50—75 mg%).

In the control experiments the response to the first stimulus of a group was closely related to the subject's estimate of attention, such that subjective attention coincided with an amplitude increase of all components. This was most marked in N_{1b}, which increased on the average by 62 % (95 % confidence interval, CI 32—92 %) of the original inattentive value (31.2 μV, 95 % CI 20.3—42.1 μV). The response to the second stimulus of a group was drastically reduced in amplitude, with N_{1b} reaching a mean of 2.9 μV (95 % CI 1.0—4.8 μV). The responses to the following stimuli of the group did not reveal a further amplitude attenuation.

Ethanol reduced the overall amplitude of the auditory evoked response mainly in the not habituated state and regardless of the subjective state of attention. So the amplitude of N_{1b} in the inattentive state was 14.3 μV (95 % CI 7.3—21.3 μV). The process of habituation was not obviously disturbed in the sense of being delayed or incomplete. There was, however, an increase in latency in all states and for all components except P_{2a}.

From these results it is obvious that even a small dose of ethanol may delay and depress the unspecific auditory evoked response in man. This phenomenon cannot solely be explained as a result of a decrease in vigilance (Fruhstorfer and Bergström 1968). It is possible, that additionally a peripheral attenuation of the afferent sensory information analogous to a decrease in stimulus intensity occurs (Rapin et al. 1966).

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References

Davis, P. A., F. A. Gibbs, H. Davis, W. W. Jetter and L. S. Trowbridge: Quart. J. Stud. Alcohol 1941, 1, 626—637.
Fruhstorfer, H. and R. M. Bergström: in preparation.
Gross, M. M., H. Begleiter, M. Tobin and B. Kissin: J. Nerv. ment. Dis. 1965, 143, 152—158.
Holmberg, G. and S. Mårtens: Quart. J. Stud. Alcohol 1955, 16, 411—424.
Rapin, I., H. Schimmel, L. M. Tourk, N. A. Krasnegor and C. Pollak: Electroenceph. clin. Neurophysiol. 1966, 21, 333—344.

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On the Use of Correlograms of Short EEG Samples in EEG Vigilance Studies

By

\ Harkinen and E. Veikamaki

It can be seen in correlograms of EEG that the periodicity typical for wakefulness disappears during sleep (Brazier 1960) According to Daniel correlogram ratios are useful in quantification of EEG arousal (Daniel 1964 1965)

The longer the EEG sample the more reliable is generally the correlogram, However if we want to measure changes in vigilance short EEG samples should preferably be used. In the present work correlograms of short EEG samples were used to investigate vigilance quantification in humans. The data thus obtained were compared with those of visual analysis.

Five healthy men (20—30 years) with alpha EEG during wakefulness volounteered for these experiments. An average of 430 EEG samples of two second duration were analysed for each subject. The EEG was recorded from four midsagittal electrodes, stored on analog tape and written on paper Visual analysis was done according to ntes classification (Bente 1965) For computer analysis the occipital EEG was entered into a laboratory computer (u-Line) with a sampling rate of 8 msec The computer calculated the autocorrelogram function (Hannan 1967) in 30 sec

The difference between the first minimum and maximum (C1) the main component in Daniel' Synchronization Ratio was calculated by the same computer from the correlogram. In addition th difference between the second minimum and maximum (C2) was also calculated. Because of the short sampling time there may sometimes be periodicity in correlograms even during sleep. Therefore the d ration of the first period (P) of the f nction was also determined. The ratio P/(C1+C2) indicates autocorrelogram index (ACI)

ACIs were grouped acc rding to isual analy is. All data were treated together although they were recorded from different subjects nd in different times Th groups were tested for signific nce by Student t test.

Highly significant differences were found between the phases A2 A3 A4 and B1 The phases B2, C and D differed only by the probability of about 80 % In general ACI seems to be less reliable as far as ts higher alues are concerned just a is the case d ring sleep.

The results showed that correlograms of short EEG samples are not alone reliable enough for quantification of sleep.

It is natural that this method cannot quantify phenomena such as sigma rhythm. It is useful, however for quantification of different phases of alpha activity

References

B[illegible], D Habilitationsschrift, Erlangen 1963
Brazier, M. A. B., *Electroenc ph. Cli. Neurophysiol.* Suppl. 13 1960 247—356
D[illegible], R. S *Science* 1964. *145* 721—723
D[illegible], R. S *Prog. phys. ology* 1963. *2* 146—160.
H[illegible], E. J Time series analysis. London 1967

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Electrical Excitability of the Ampullae of Lorenzini in the Ray

By

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It was shown previously (Waltman 1966) that the ampullary canal of Lorenzini, an electric receptor in elasmobranchs, behaves like an ideal submarine cable terminated by the impedance of the sensory epithelium in the wall of the ampulla. Furthermore, there was some indication that the ampulla, unlike the canal, might be excitable This has been confirmed in excised hyoid ampullae from *Raja* species.

The transected neck of an ampulla was insulated by sucrose gap or by vaseline seals and negative series feedback (Frankenhaeuser 1957) Th potential difference across the high resistance so obtained and hence cross the ampullary wall, was recorded in the sense external potential minus potential in th lumen. Under these conditions, long trains of all-or-nothing positi e-going action potentials were discharged across the ampullary wall, either spontaneously or in response to weak positive currents (*i.e* to a cathode in the lumen) The rheobase for a silent ampulla was about 2 nA. The action potentials reached 60 mV in amplitude and had a shape like nerve membrane action potentials but with a time scale 100 times slower (risetime about 50 msec t 10° C) A burst of afferent impulses coincident with the front of each 'action potential' could be led from single axons n the ampullary nerve

When the transmural currents were then measured nder oltage clamp conditions, the response to tep polarizations was graded When the transmural potential was displaced from zero in negative potential steps, the currents after the capacitative surge were small and negative transmural resistance eraging 300 kΩ For positive steps, from zero to about 40 mV the currents grew larger were initially negati but reversed direction t longer times. The initial currents were positive for positive steps greater than about 40 mV A continuous relation between transmural current and voltage was obtained that was similar to that of the nerve membrane

If it is the receptor cells i the ampullary wall that are excit bl their plasma membranes should beha asymmetrically since only positi currents were excitatory If the lumenal surface of a receptor cell were ctive and the basal surface

passive the latter (which bears the afferent synapses) would be depolarized by negative currents.

The input of the ampullary canal *in vivo* is apparently shunted by a low impedance (Waltman 1966). In this condition the potential difference across the ampullary impedance and the low resistance in series with it would be "clamped" at the voltage between the base of the ampulla and the pore end of the canal at the skin surface. Changes in this voltage should then evoke graded rather than regenerative responses in the ampullary wall.

This investigation was supported by the Swedish Natural Science Research Council.

References

Frankenhaeuser, B. *J. Physiol. (Lond.)* 1957. *135*. 550—559.
Waltman, B. *Acta physiol. scand.* 1966. *66*. Suppl. 264.

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The Effect of Procaine on the Lowly Adapting Stretch Receptor Neurone of Lobster

By
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Biphasic Action of Testosterone on Muscle Glycogen Synthetase

By
S. Adolfsson and K. Ahrén

It is highly probable that glycogen synthesis is regulated by changes in activity of the synthetase enzyme (UDP-glucose α-1,4-glucan α-4-glucosyl-transferase). This enzyme exists in two forms: one which is dependent on glucose-6-P for its activity (D-form) and another which is independent of glucose-6-P (I form). These forms are interconvertible. Enzyme activity is increased when D-form is converted to I-form.

Insulin is known to stimulate glycogen synthesis and it has been shown (*e.g.* Villar Palasi and Larner 1961) that this hormone also produces D $\rightarrow$ I transformation in

TABLE I. Effects of testosterone on glycogen synthesis in th levator ani muscle of immature male rats.

Hours after injection of testosterone	Glycogen mg/g wet tissue		Glucose-^{14}C CPM/µg glycogen		Synthetase % I-form[a]	
	Control	TP	Control	TP	Control	TP
10	[illegible]6 ± 0.3	3 6 ± 0 1	51 ± 5	197 ± 30	14.2 ± 1.2	25.4 ± 3 1
	$p < 0.02$		$p < 0.005$		$p < 0.05$	
24	2.9 ± 0.1	7 1 ± 0.5	15 ± 2	192 ± 9	13.2 ± 0.7	8.3 ± 0.2
	$p < 0.001$		$p < 0.001$		$p < 0.001$	

Testosterone propionate (TP) was injected intramuscularly (100 mg/kg b.w.) There are 3—4 rats in each group. Mean values ± S.E. are given. Methods and calculations ha been described earlier (for ref. see Sovik and Adolfsson 1968)

Ten nd 24 hrs after the injection of TP the muscles were isolated and incubated for 2 hrs in Krebs-HCO_3 buffer in the presence of glucose ^{14}C. Incorporation of radioactiv ty into glycogen was measured.

A crude muscle extract was used. The synthetase activity assayed in the absence of glucose-6-P (I-form) is given in per cent of the activity in the presence of this ester (I + D forms)

the synthetase enzyme system. It has not, however been possible to see this effect of insulin during every condition when insulin is known to stimulat glycogen synthesis (e.g. Sovik 1966) The physiological significance of hormonal influences on the synthetase system apparently needs to be further elucidated. Experiments ha e there fore been started in an effort to study the effects of another hormone iz. testosterone on glycogen synthesis and on the synthetase enzyme.

The levator ani muscle of immature male rats has been used as the test organ. It has previously been shown that testosterone markedly timulates glucose uptake in this muscle (Arvill 1967) As can be seen from T ble I th glycogen content of this muscle was increased both 10 and 24 hrs after an injection of testosterone propionate (TP) Ten hours after the injection there was also an increase in the I form of the synthetase enzyme while 24 hrs after the injection the I form was decreased. As indicated by the incorporation of glucose- ^{14}C nto glycogen, the rate of glycogen synthesis was, however of the same magnitude 10 and 24 hrs after the injection of the hormone

Testosterone thus seems to h a biphasic action on the synthetase enzyme y tem. Initially there is an activation by means of a D → I transformation, the ncrease in I form is subsequently diminished and reduced below the control level. As th rate of glycogen sy thesis is not concomitantly decreased, this indicates that there must be an add tional phy iological mechanism other than the D → I transformation alone in the sy thetase enzyme system, to explain the prolonged timulation of glycogen synthesis after njection of testosterone

References

Ar ill, A., Act dec (Kbh) 1-6 Suppl. 122, 1—14
Sovik, O Act physiol sc 4 1966 68 246—254
Sovik, O nd S Adoif son Life Sci. 1968, 7 Part II 549—55
Villar-Pala i, C. and J Lar n, A h. Biochem 1961 94 436—44

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The Relationship between Uptake and Metabolic Action of Insulin in Isolated Fat Cells

B

J Gliemann

Fat cells isolated from rat epid'dymal fat pads by treatment with collagenase were incubated at 20 C in glucose free Krebs-Ringer bicarbonate buffer containing 10 μ L of insulin per ml. Aliquots of the cell suspension were removed at time intervals. Subsequent assay of the incubation medium by the isolated fat cell method showed the cells had removed 111 ± 19 μL of insulin per 100 mg of cell triglyceride uring the first 4 min of incubation. There was no further uptake of insulin during the following 20—30 min whereafter a second phase of uptake was observed. Cells which were exposed to insulin 10 μL/ml) for 5 min, washed repeatedly and reincubated in buffer containing insulin, howed a similar rapid uptake of insulin.

Some cells were removed at the end of the period of initial insulin uptake and incubated at 37 C in buffer ontaining 0 1 mg of glucose per ml and an excess of insulin antibodies The glucose uptake and the conversion of glucose 1 ^{14}C t $^{14}CO_2$ and ^{14}C-triglycerides rose t a maximal level within 5—10 min, stayed maximally stimulated for about 30 min nd finally declined to a basal level within a period of about 15 min. The return t a basal glucose uptake thus coincided with the second period of insulin uptak by cells incubated in the continuous presence of insulin. Cell which had been incubated f r 10 mi in the presence of insulin showed immediately a maximal uptake and metabolism of glucose upon transfer to glucose containing buffer at 37° C.

The following model is suggested for the initial interaction between insulin and the cell Insulin is attached to a receptor presumably at the cell membrane This event does not *per se* increase the glucose transfer but triggers (a) secondary process(es) which in turn augments the gl ose influx This change of the cell membrane persists for about 30 min, even though insulin may be removed from the receptors. Finally the cell membrane returns to its previous state nd reconstitutes the barrier to glucose influx.

of histamine dihydrochloride to a concentration of 10 eq/l every 45th min gave the most stable system. A good nutritional state of the animals was of the utmost importance. It was found necessary to feed the animals manually 2—3 times a week. The isolated frog mucosa has a constant hydrogen ion production and electrogenous properties for 4 hrs or more under these conditions (hydrogen ion production 3—6 μeq/hr cm^2 voltage clamp current 1—2 μeq/hr cm^2 and potential difference 10—30 mV)

The effects of hypoxia upon voltage clamp current and hydrogen ion production were quite different. Reduction of pO_2 caused a successive decrease in the hydrogen ion production while voltage clamp current and the electric potential difference were maximal at a pO_2 of about 300 mm Hg This dissociation between hydrogen ion transport and voltage clamp current under hypoxic conditions might be explained as the active transport of positive ions from the mucosal to the serosal side.

References

C [illegible], J T and B. E. V [illegible] *Biochim. Biophys. Act* (Amst.) 1965 *94* 280—292.
Hogben, C. A. M., *F d P oc.* 1965. *24* 1353—1359
Kitahara, S., *Amer J Physiol.* 1967 *213* 819—823
Kitahara, S., K. R. Fox and C. A. M. Hogben, Abstracts of the Eleventh Annual Meeting of the Biophysical Society 1967 p. 49

C 29

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Behaviour of Whole Rat Thyroid when I^{1} is Offered in vitro or during Electrophoresis

By

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Paper electrophoresis has been found to be the most effective means for separating inorganic from organic iodine in the thyroid (Nakatagi and Inbar 1963) The aim of *present investigation was to study whether whole rat thyroid lobes could be used* for in vitro studies on electrophoresis paper and whether under these conditions, the cells still retain some of their physiological functions (Mack 1966)

The experiments were divided into two main parts. Th first part consisted of lobes incubated for 2 hrs with I ^{131} and thyrotropin or propylthiouracil, then placed on a paper strip and submitted to electrophoresis (Veronal buffer 300 V 2 hrs) whereupon I ^{131} and radioactive proteins leaked out on the paper forming th peaks $R_f = 0.16$ and $R_f = 0.41$ behind the I $R_f = 1.0$

In the second part the lobes were incubated for 30 min with thyrotropin or propylthiouracil, before being placed on the paper strip. Then the I was pipetted 15 mm cathodally of the lobes. During 2 hrs electrophoresis radioactive proteins formed one peak. $R_f = 0.46$, behind the I $R_f = 1.0$

These results indicate that a narowing of the unit's response area appears when the rate of frequency change of the stimulus increase up to a certain value. The conclusion is that the spectral resolution increases within a certain range of sweep speeds.

This type of performance cannot be accomplished solely by filters made up of linear elements such as those which are represented by the properties of the basilar membrane. The response of such filters to sweep tones has a constant amplitude up to a certain sweep rate, above which the response spreads out in time and its amplitude decreases. It is assumed that the mutual interactions betwene excitatory and inhibitory response areas play an important role in accomplishing the characteristics demonstrated.

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Evolutionary Changes in Tissues Stores and Vascular Actions of Histamine in Vertebrates

By

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The vascular actions of histamine and the occurrence of this amine in tissues have been systematically studied in one or more species from each subclass of living lower vertebrates. Pharmacological responses were recorded as changes in systemic blood pressure and as changes in perfusion pressure in artificially perfused preparations. Tissue levels and cellular localization of histamine were determined by methods utilizing the fluorescence of the condensation product of histamine with o-phthalaldehyde. The results indicate that storage of histamine in the mucosal layer of the digestive tract (non-mast cell histamine) is a feature which was present already in primitive jawed vertebrates. This feature has been maintained in all vertebrates with normal stomach functions, but is absent in vertebrates where the stomach has later on become reduced (some species of fish). Storage of histamine in loose connective tissue (in the mast cells) represents a more recent development. Mast cell histamine is found in all studied descendants of primitive reptiles indicating that it had already appeared in this group of vertebrates. The same feature seems to have developed independently among the immediate ancestors of lungfish. Lungfish and descendant of primitive reptiles are also the only vertebrates which show high vascular sensitivity to histamine. The different steps in the evolution of tissue histamine stores and vascular actions of histamine in vertebrates are suggested in Fig. 1.

With respect to the functional significance of mast cell histamine the results direct the attention towards physiological mechanisms common to lungfish and primitive reptiles.

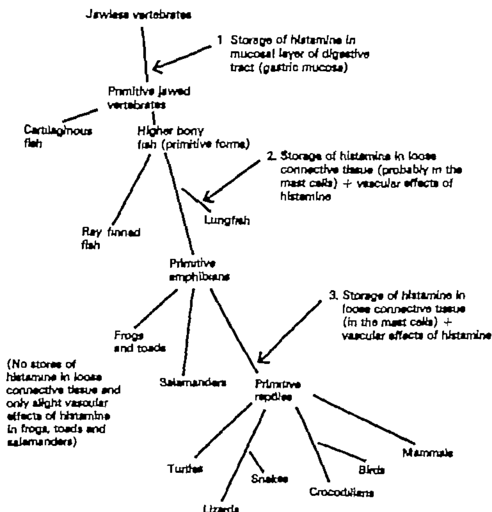


Fig. 1 A simplified diagram of the evolution of different ertebrates, with arrows (marked 1 2 and 3) indicating historical occasions in relation to the pharmacology of histamine.

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Adenine Nucleotides and Catecholamine Mobilization in Adrenal Medulla and in Sympathetic Nerves

By

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The mechanism for excitation transmitter mobilization coupling" in sympathetic nerves is at present poorly understood. On the other hand important progress has recently been made concerning the corresponding mechanism for hormone secretion from the adrenal medulla The demonstration of two major constituents of the catecholamine storage granules, adenine nucleotides and specific protein, appearing in the effluent from the stimulated gland concomitantly with the catecholamines and in about the same quantitative relationship in which they occur in the amine storage granules, has led to the conclusion that the hormones are secreted directly from the granules. The additional finding that the major n cleotide in the gran les, ATP may under certain conditions appear in the perfusate in undegraded form has been interpreted as evidence that the mechanism for this extrusion of granular material is exocytosis (Douglas 1966)

These findings in the adrenal gland have led to speculations concerning the applicability of the same principle to sympathetic nerves, i.e transmitter liberation by exocytosis. However no experimental evidence for or against this possibility seems to have appeared as yet

The present experiments were designed to study this issue by determining whether adenine nucleotides from the amine storage granules in adrenal medulla and in sympathetic nerves appear n the effluent from the acetylchol ne stimulated, perfused adrenal medulla and from the perfused spleen stimulated electrically by its nerves.

The method used consisted in labelling nonselecti ely the adenine nucleotides in adrenal medulla and sympathetic nerves of the cat by prolonged treatment with radioactive phosphorus (P32) and in subsequent determination of labelled adenine nucleotide material in the effluent f om the isolated perfused adrenal gland and spleen. The outflow of catecholamines was monitored by determining the tritium content of the effluent after labelling the amine tores in gland and nerves by per fusion with tritium-labelled noradrenaline

On secretion stimulation by inf sion of acetylcholine P32 labelled material consistently appeared in the effluent from the adrenal gland concomitantly w th the amines. In the spleen no such correlation between outflow of amine and P32 labelled material was observed in response to electrical nerve stimulation, when care was taken to prevent contamination with blood.

The results support the exocytosis hypothesis for adrenal medullary secretion, but seem to provide for the first time experimental evidence that "excitation-transmitter mobilization coupling" in sympathetic nerves is based on other mechanisms.

References

DOUGLAS W W *Pharmacol Rev* 1966, *18* 471—480

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The Disappearance of Radio-Isotopes in Skeletal Muscle of the Dog Following Sympathetic Vasodilator Nerve Stimulation

By

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The sympathetic cholinergic vasodilator nerves to the hind limb of anesthetized dogs were electrically activated either from the hypothalamus or by lumbar sympathetic chain stimulation after blocking the vasoconstrictor nervous response with dihydroergotamine. The disappearance or clearance, from a radioactive depot of either ^{133}Xe, Na ^{131}I or ^{22}NaCl, injected into the gastrocnemius muscle, was determined, during resting conditions and during the sympathetic vasodilatation, by a scintillation detector and two scalers using counting periods of 12 sec. The gastrocnemius muscle was in some experiments isolated and the blood flow through a cannulated vein or artery determined by a drop counter. In another type of experiments the muscle was left intact and the blood flow to the entire hind limb was measured by an electromagnetic flowmeter probe around the femoral artery. The vasodilatations produced by stimulation were usually transient, lasting from 36 to 72 sec, thus allowing three to six counting periods. When the muscle was left intact vasodilator nerve stimulation led to an enhancement of the blood flow and an increased disappearance of all three isotopes used. In the experiments with the muscle isolated, the sympathetic vasodilatation was paralleled by an increased disappearance of ^{133}Xe and Na ^{131}I. In some animals, however, when the experiment had been running for some time during which fluid loss from the prepared tissues was inevitable, vasodilator nerve stimulation would lead to an increased blood flow with no detectable change in the disappearance. This finding was preferably observed when Na ^{131}I was used.

The results indicate that the transcapillary passage of solutes in the skeletal muscle measured as isotope disappearance is enhanced during activation of the sympa-

thetic cholinergic vasodilator nerves in the intact dog. This increased disappearance following sympathetic vasodilatation will be obscured after prolonged or traumatic preparation procedures of the muscle. This latter finding may be one explanation why Hyman *et al.* (1959) did not notice any change in the clearance rate of $Na^{131}I$ upon hypothalamic vasodilator nervous outflow stimulation in isolated hind limb muscles of the cat.

References

Hyman, C., S. Rosell, A. Rosell, R. R. Sonnenschein and B. Uvnäs, *Acta physiol. scand.* 1959. 46. 358—374